Diversity and Function of the Microbial Community on Anodes of Sediment Microbial Fuel Cells fueled by Root Exudates

Doctoral thesis

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Anode microbial communities are essential for current production in microbial fuel cells. Anode reducing bacteria are capable of using the anode as final electron acceptor in their respiratory chain. The electrons delivered to the anode travel through a circuit to the cathode where they reduce oxygen to water generating an electric current. A novel type of sediment microbial fuel cell (SMFC) harvest energy from photosynthetically derived compounds released through the roots. Nothing is known about anode microbial communities of this type of microbial fuel cell.

This work consists of three parts. The first part focuses on the study of bacterial and archaeal community compositions on anodes of SMFCs fueled by rice root exudates. By using terminal restriction fragment length polymorphism (T-RFLP), a profiling technique, and cloning / sequencing of 16S rRNA, we determined that the support type used for the plant (vermiculite, potting soil or rice field soil) is an important factor determining the composition of the microbial community. Finally, by comparing microbial communities of current producing anodes and non-current producing controls we determined that *Desulfobulbus*- and *Geobacter*-related populations were probably most important for current production in potting soil and rice field soil SMFCs, respectively. However, δ-proteobacterial *Anaeromyxobacter* spp., unclassified δ-proteobacteria and Anaerolineae were also part of the anode biofilm in rice field soil SMFCs and these populations might also play a role in current production. Moreover, distinct clusters of *Geobacter* and *Anaeromyxobacter* populations were stimulated by rice root exudates. Regarding Archaea, uncultured Euryarchaea were abundant on anodes of potting soil SMFCs indicating a potential role in current production. In both, rice field soil and potting soil SMFCs, a decrease of *Methanosaeta*, an acetotrophic methanogen, was detected on current producing anodes.

In the second part we focused our study on identifying the bacteria capable of rice root exudate assimilation on anodes of planted SMFCs. Using stable isotope probing (SIP) with ^{13}C-CO_2 combined with high throughput sequencing, we detected that labeled bacteria belonged to β-proteobacteria and Anaerolineae indicating their relevance in root exudate degradation. The main current producing bacteria, belonging to δ-proteobacteria were not able to assimilate root exudates. A microbial “food chain” combining activities of anode reducing bacteria with root exudate degrading bacteria is necessary for current

production. However, we cannot dismiss the possibility that some bacteria might be able to directly use root exudates for current production.

In the last part, we found that by submerging an anode into rice field soil up to 50% methane emission was reduced compared with open circuit controls. This mitigation could not only be explained by competition for common electron donors like acetate. We suggest that the anode, even in non-current controls, can be used as electron acceptor capturing electrons and transferring them from one part of the sediment to a spatially distant one, communicating biogeochemical processes occurring in different parts of the sediment.

Our work is a first approach in understanding the microbial diversity on anodes of SMFCs fueled by rice root exudation and their potential as methane emission mitigation strategy.

Voraussetzung für einen Stromfluss in Bio-Brennstoffzellen (*microbial fuel cell*; MFC) ist die Besiedlung der Anode durch mikrobielle Gemeinschaften. Anoden-reduzierende Bakterien sind in der Lage, die Anode als terminalen Elektronenakzeptor in ihrer Atmungskette zu nutzen. Die an der Anode abgegebenen Elektronen fließen in einem Stromkreis zur Kathode, an der Sauerstoff durch Aufnahme der Elektronen zu Wasser reduziert und ein elektrischer Strom erzeugt wird. Ein neuer Typ von mikrobiellen Brennstoffzellen im Sediment (*sediment microbial fuel cell*; SMFC) nutzt die Energie von photosynthetisch assimilierten Verbindungen, die von Reiswurzeln ausgeschieden werden, wobei die Zusammensetzung der mikrobielle Gemeinschaft auf der Anode bisher nicht bekannt war.

Die vorliegende Arbeit besteht aus drei Teilen. Der erste Teil beschäftigt sich mit der Analyse der Zusammensetzung bakterieller und archaeeller Gemeinschaften auf der Anode von Boden-basierten mikrobiellen Brennstoffzellen, die durch Reiswurzelexsudate angetrieben werden. Durch Anwendung von terminalem Restriktionsfragmentlängenpolymorphismus (T-RFLP), Klonierung und Sequenzierung der 16S rRNA fanden wir heraus, dass das Trägermaterial für Pflanzen (Vermikulit, Pflanzerde oder Reisfeldboden) ein wichtiger Faktor für die Zusammensetzung der mikrobiellen Gemeinschaft ist. Beim Vergleich der mikrobiellen Besiedlung von stromerzeugenden Anoden und nicht-stromerzeugenden Kontrollen fanden wir *Desulfobulbus*- und *Geobacter*- verwandte als wahrscheinliche Hauptproduzenten in auf Pflanzerde und Reisfeldboden basierten Biobrennstoffzellen (SMFC). Allerdings waren auch zu den δ-Proteobacteria gehörende *Anaeromyxobacter* spp., nicht-klassifizierte δ-Proteobacteria und Anaerolineae im Biofilm der Anode von Reisfeldboden basierten Biobrennstoffzellen vertreten und könnten ebenfalls eine Rolle in der Stromerzeugung spielen. Darüber hinaus wurden bestimmte Gruppen von *Geobacter* and *Anaeromyxobacter* durch Reiswurzelexsudate stimuliert. Bezüglich der Archaea waren nicht-kultivierbare Euryarchaea auf der Anode von mikrobiellen Brennstoffzellen mit Pflanzerde nachweisbar, was auf eine potentielle Rolle dieser Population in der Stromerzeugung hindeutet. In mikrobiellen Brennstoffzellen basierend auf Reisfeldboden oder Pflanzerde nahm der Anteil von Sequenzen der *Methanosaeta*, einer Gattung acetotropher methanogener Archaea, an stromerzeugenden Anoden ab.

Im zweiten Teil konzentrierten wir unsere Arbeit auf die Identifizierung der Bakterien, die zum Abbau von Reiswurzelexsudaten an der Anode von bepflanzten boden-basierten mikrobiellen Brennstoffzellen befähigt sind. Mittels stabiler Isotopenbeprobung (*stable isotope probing*; SIP) mit $^{13}C\text{-}CO_2$ kombiniert mit Hochdurchsatzsequenzierung konnten wir ^{13}C-markierte Bakterien zugehörig zu den β-Proteobacteria und Anaerolineae als mögliche relevante Mikroorganismen beim Abbau von Wurzelexsudaten ausmachen. Die hauptsächlich für die Stromerzeugung verantwortlichen Bakterien zugehörig zu den δ-Proteobacteria waren nicht markiert. Diese Daten deuten darauf hin, dass bei der Umsetzung von Wurzelexsudaten eine mikrobielle anaerobe „Nahrungskette“ aktiv war, die sich aus Exsudat-abbauenden und Anoden-reduzierenden Bakterien zusammensetzte; beide trophischen Gruppen scheinen für die Stromerzeugung unerlässlich zu sein. Allerdings können wir nicht ausschließen, dass einige Bakterien möglicherweise Wurzelexsudate direkt zur Stromerzeugung nutzen können.

Im letzten Teil der Arbeit fanden wir heraus, dass in geflutetem Reisfeldboden durch die Anwesenheit einer Elektronen-akzeptierenden Anode 50% weniger Methan emittiert wurde als in der Kontrolle (mit offenem Stromkreis). Diese Abnahme konnte jedoch nicht nur durch eine Konkurrenz um gewöhnliche Elektronendonatoren wie Acetat erklärt werden. Wir vermuten vielmehr, dass die Anode, selbst in nicht-stromerzeugenden Kontrollen, als terminaler Elektronenakzeptor genutzt wird um Elektronen einzufangen und diese von einem bestimmten Teil des Sedimentes zu einem räumlich distinkten Bereich zu übertragen. Möglicherweise verbindet die Anode als Elektronenleiter biogeochemische Prozesse, die in räumlich getrennten Bereichen des Sediments auftreten, wodurch eine Unterdrückung der Methanbildung erklärt werden könnte.

Unsere Arbeit ist ein erster Ansatz um die mikrobielle Diversität der Anoden-Biofilme von Boden-basierten mikrobiellen Brennstoffzellen, angetriben durch Reiswurzelexsudationen zu verstehen und ihr Potential zur Verringerung der Methanemissionen zu ergründen.

Chapter 1

General Introduction

1.1 Microbial fuel cells

History of microbial fuel cell development

At present most of the energy demand is supplied by fossil fuels and nuclear sources (Dresselhaus & Thomas, 2001, Armaroli & Balzani, 2007). The use of stored carbon in fossil fuels is increasing the carbon dioxide concentration in the atmosphere, from approximately 280 parts per million (ppm) in pre-industrial times to 382 ppm in 2006 (Intergovernmental Panel on Climate Change - IPCC, 2007). Present CO_2 concentrations are higher than any time in at least the last 650,000 years (IPCC, 2007). As CO_2 is a greenhouse gas, the accumulation in the atmosphere is one of the factors causing an increase in global average air and ocean temperatures, which causes widespread melting of snow and ice and rising global average sea level (IPCC, 2007). These environmental problems caused by the use of fossil fuels have driven the search for new alternative energy sources such as solar energy and wind energy. In this context, microbial fuel cells (MFCs) have emerged as a promising yet challenging technology. In a MFC, microorganisms convert chemical energy present in organic compounds directly into electric energy by transferring electrons to an anode. The earliest work on MFC dates back to 1911 where Potter (1911) described the production of electric energy from living cultures of *Escherichia coli*. His work did not receive any considerable attention until 1931, when Cohen (1931) was able to produce a voltage larger than 35 V from MFCs connected in series. MFCs became popular in the 1960s, when the National Aeronautics and Space Administration (NASA) in USA carried out further research to

assess their application in space missions. However, relatively little was understood about how these MFCs functioned and about fuel oxidation. New insight came from the studies by Allen and Bennetto in the 1980-90s (Allen & Bennetto, 1993) who discovered that current density and power output could be greatly enhanced by using electron mediators to accelerate the electron transfer rate from microorganisms to the anode surface. The next significant advance occurred when some microorganisms were found to transfer electrons directly to the anode without the need of mediators rendering MFCs as a viable technology to generate electric power (Kim*, et al.*, 1999, Chaudhuri & Lovley, 2003).

The interest in MFCs has tremendously grown in recent years; Figure 1 shows the increase of research articles based on a search of the term "Microbial fuel cell" in the citation database Web of Science and indicates an increase of almost 60-fold in the last decade (1998-2009). Moreover, an increase in 5 orders of magnitude of the power density produced my MFCs has been observed in 10 years of research (Logan & Regan, 2006a). The current power density output trends are encouraging but power density levels should increase substantially to render MFC technology feasible for commercial applications. MFC technology can be combined with several biotechnological processes for example with wastewater treatment (Oh & Logan, 2005, Heilmann & Logan, 2006, Rodrigo*, et al.*, 2007, Feng*, et al.*, 2008, Lu*, et al.*, 2009, Patil*, et al.*, 2009, Wang*, et al.*, 2009, Pant*, et al.*, 2010), aiding in bioremediation of hydrocarbons in groundwater and sediment (Morris & Jin, 2008, Zhang*, et al.*, 2010) or as power sources for environmental sensors (Kang*, et al.*, 2003, Williams*, et al.*, 2010) which increases the application niches of MFCs.

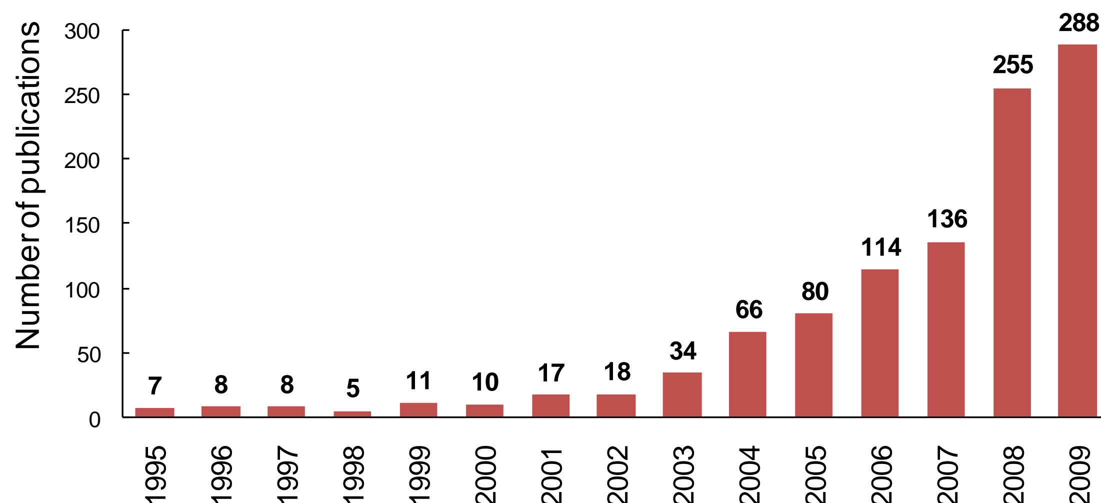

Figure 1 The number of publications on microbial fuel cells. The data is based on the number of articles mentioning "microbial fuel cell" in the citation database Web of Science from the year 1995 to 2009.

Microbial fuel cell principle

In microbial fuel cells (MFC), microorganisms oxidize organic compounds as part of their energy metabolism and transfer electrons to an anode, which acts as the terminal electron acceptor of the respiratory chain, in the process called respiration (Rabaey & Verstraete, 2005). Respiring microorganisms can use a large variety of different electron acceptors, ranging from oxygen, nitrate, iron and manganese oxides to sulfate (Madigan, *et al.*, 2000). Anode reducing bacteria are capable of using an anode as electron acceptor and gain energy from their metabolism due to the potential difference between for example NADH ($E°'$ = -320 mV for $NAD^+ + H^+ + 2e^- \longrightarrow NADH$) and cytochrome c ($E°'$ = +254mV for cytochrome c(Fe^{3+}) + e- $\longrightarrow$ cytochrome c(Fe^{2+})), whereas the MFC could be used to recover energy from the potential difference between cytochrome c ($E°'$ = +254mV) and oxygen ($E°'$ = +840mV for $O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$) (Rabaey & Verstraete, 2005, Logan & Regan, 2006a). The total potential difference would be approximately 1.2V (574mV (NADH and cytochrome c) and 586mV (cytochrome c and oxygen)). Fermentative bacteria are also able to produce current in microbial fuel cells, however, only one third of the electrons are possibly available for

electricity generation whereas two thirds remain in the produced fermentation products such as acetate and butyrate (Logan, 2004); the transfer of electrons to the anode is probably mediated by hydrogenases situated in the membrane surface (McKinlay & Zeikus, 2004).

After the transfer of electrons to the anode by bacteria, the electrons flow through a circuit to the cathode where they react with O_2 to form H_2O (Figure 2) (Rabaey & Verstraete, 2005). Concomitantly, protons diffuse through a proton exchange membrane to the cathodic chamber to preserve electroneutrality (Logan & Regan, 2006a). In this way, MFC allows harvesting electrical current from microbial activity.

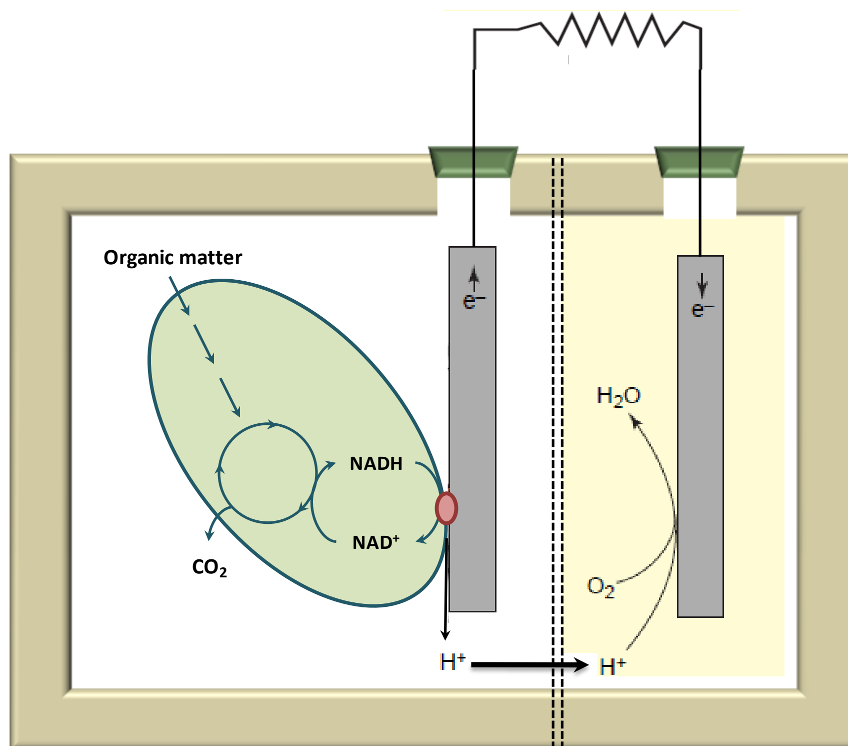

Figure 2 Scheme of a microbial fuel cell. Organic compounds are oxidized by bacteria which transfer electrons to an anode. Anodes travel through an electrical circuit to the cathode where oxygen is reduced to H_2O. Adapted from (Rabaey & Verstraete, 2005).

The direct transfer of electrons from the bacteria to the anode is hampered by overpotentials which cause a loss in the actual current output that can be obtained by MFCs. Overpotentials are potential losses due to electron transfer resistances and internal resistances (Rabaey & Verstraete, 2005). The potential losses can be classified into: activation overpotentials, ohmic losses and concentration polarization (Rabaey &

Verstraete, 2005). For MFCs, the activation overpotential seams to be the major factor in reducing the current production. The activation overpotential is caused by the activation energy needed by the bacteria to oxidize a compound at the anode surface (Rabaey & Verstraete, 2005). This overpotential can be decreased for example by adding a catalyst to the electrode surface (Park & Zeikus, 2003) or a mediator compound to the anodic solution (Park*, et al.*, 2000, Choi*, et al.*, 2003).

Microbial fuel cell configurations

A typical microbial fuel cell consists of two compartments, one anodic chamber and one cathodic chamber separated by a proton exchange membrane (PEM). Nevertheless, several other types of MFCs have been developed, for example single-chamber MFCs and sediment microbial fuel cells (SMFC).

Two-chamber and single-chamber MFCs

The simplest and widely used MFC is the two-chamber MFC (or H-shape MFC) consisting usually of two chambers separated by a proton exchange membrane such as Nafion (Park & Zeikus, 1999, Bond*, et al.*, 2002, Logan*, et al.*, 2005, Min*, et al.*, 2005) or by a plain salt bridge (Min*, et al.*, 2005). The key to this design is to choose a membrane that allows protons to diffuse from the anodic chamber to the cathodic chamber but not the organic substrate nor oxygen from the cathodic chamber to the anodic chamber. The design has some major drawbacks which limit the power generation; examples are a large distance between the electrodes and a low membrane/anode surface ratio (Oh*, et al.*, 2004). However, H-shape systems are acceptable for basic parameter research, such as examining power production using new materials (Du *et al*, 2007), types of microbial communities that arise during the degradation of specific compounds (Table 2) and can generally be autoclaved which allows the study of pure cultures (Table 1) (Logan*, et al.*, 2006).

In single-chamber MFCs, the cathode is placed in direct contact with air either in the presence or absence of a membrane. Much larger power densities have been achieved using oxygen as the electron acceptor when aqueous-cathodes are replaced with air-cathodes (Du*, et al.*, 2007). In the simplest configuration, the anode and cathode

are placed on either side of a tube, with the anode sealed against a flat plate and the cathode exposed to air on one side, and water on the other (Liu & Logan, 2004, Liu*, et al.*, 2004, Liu*, et al.*, 2005). Two-chamber and single-chamber MFCs have been usually used with simple organic substrates or complex substrates like wastewaters (Pant *et al*, 2010). The most interesting and economically feasible application would be electric energy production from the degradation of organic matter in wastewaters combining in this way current production and waste degradation.

Sediment microbial fuel cells

Energy can be harvested from organic matter in aquatic sediments in so called sediment microbial fuel cells (SMFC) (Reimers*, et al.*, 2001, Tender*, et al.*, 2002). They consist of an anode embedded in anoxic sediments connected to a cathode suspended in the overlying aerobic water (Lovley, 2006). In these systems no addition of organic matter is necessary and complex organic matter from the sediments is broken down by hydrolytic and fermentative microorganisms to acetate and other electron donors. Several reactions have been implicated to contribute to the generation of electric current in SMFCs: 1) the chemical oxidation of humic acids, Fe(II) , but especially sulphur compounds at the anode, 2) the microbial oxidation of organic compounds such as acetate and 3) the microbial oxidation of S° to sulphate (Reimers*, et al.*, 2001, Bond*, et al.*, 2002, Tender*, et al.*, 2002, Holmes*, et al.*, 2004a, Ryckelynck*, et al.*, 2005). A current can be generated when the electrons released during these processes are captured by the anode and travel to the cathode where oxygen is reduced. The anode microbial communities have been studied in SMFC with a number of different aquatic sediments; marine, salt-marsh and freshwater (Bond*, et al.*, 2002, Holmes*, et al.*, 2004c). In these systems, a clear enrichment of δ-Proteobacteria was found with a predominance of the family Geobacteraceae. In marine and salt-marsh SMFC *Desulfuromonas acetoxidans* was found to be responsible for current production while *Geobacter* spp. were abundant when freshwater sediments were used (Bond*, et al.*, 2002, Holmes*, et al.*, 2004c). SMFCs have been applied to power electronic devices in remote locations, such as the bottom of the ocean (De Long & Chandler, 2002). Recently, it was demonstrated that sediment microbial fuel cells could also harvest

energy from root exudates (Figure 3) (De Schamphelaire*, et al.*, 2008, Kaku*, et al.*, 2008, Strik*, et al.*, 2008). In these particular SMFCs, a plant is included into the system and the anode is buried in the rhizospheric soil. One advantage of these type of SMFC is the higher current output obtained, compared with unplanted SMFCs, due to continuous release of organic compounds through the roots which are used by anode reducing bacteria to produce current (De Schamphelaire *et al*, 2008). De Schamphelaire *et al.* (2008) demonstrated that the presence of plants increased the power output with a factor of 7 and that it is possible to oxidize plant-derived material *in situ*. The authors stated that current production in rice planted SMFC were substantial compared to marine sediment microbial fuel cells. Microbial communities on the anodes of planted SMFCs have not been studied up to date and nothing is known about the effect of support or plant on the anode bacterial community composition. These types of SMFCs offer a potential for a sun-driven power generation for example in remote areas with high solar inputs without the need for costly construction materials (De Schamphelaire*, et al.*, 2008)

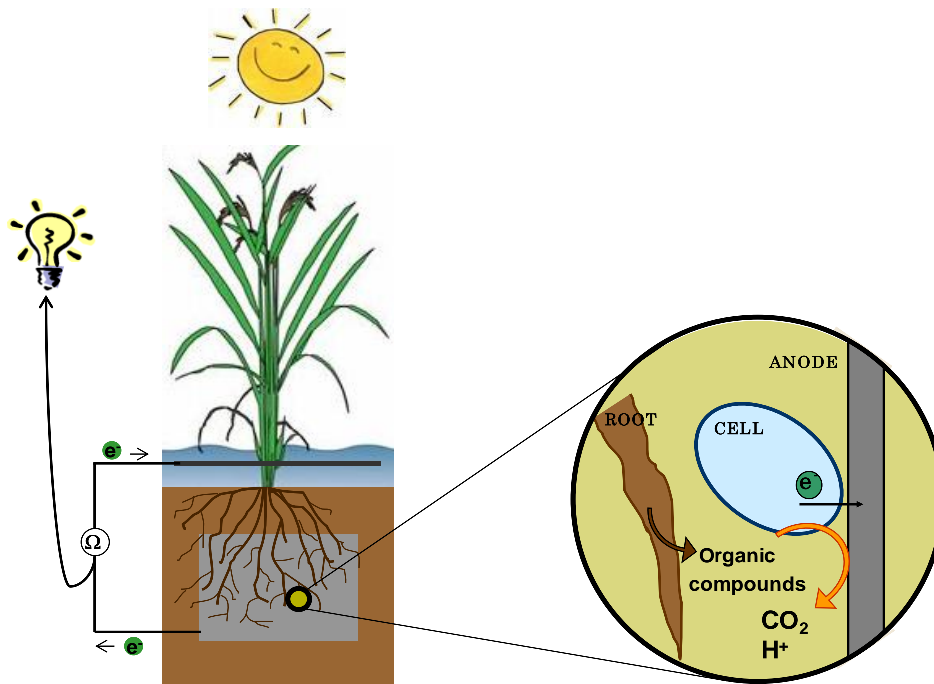

Figure 6 Scheme of SMFC fueled by root exudates. Plant fixes CO_2 and releases root exudates into the soil which are degraded by bacteria. An anode buried into the rhizospheric soil (A) connected to a cathode (B) lying in the overlaying water allows current production by electrogenic microorganisms.

Bacterial anode biofilms

The formation of a biofilm on the anode surface is essential for the efficient transfer of electrons in a MFC (Franks, *et al.*, 2010). As explained above, anode reducing bacteria are able to use the anode as terminal electron acceptor. The flow of electrons from the anode to the cathode through an electrical circuit generates an electric current. MFC research has been mainly focused on increasing power output in order to develop economically feasible systems. Even though the study of microbial communities on anodes has not been the main focus, advances have been made in understanding electron transfer mechanisms and current generation by anode biofilm microorganisms (Lovley, 2006, Rabaey, *et al.*, 2007, Lovley, 2008, Logan, 2009).

Microbial fuel cells can be operated in pure culture or in mixed culture. Pure culture MFCs are important for determining the capability of strains to produce current and to study the mechanisms of electron transfer to the anode (Kim, *et al.*, 1999, Park, *et al.*, 2001, Bond & Lovley, 2003, Chaudhuri & Lovley, 2003, Pham, *et al.*, 2003, Holmes, *et al.*, 2004b, Reguera, *et al.*, 2006, Prasad, *et al.*, 2007, Ringeisen, *et al.*, 2007). Up to date, 29 strains have been shown to produce current in pure culture belonging to all classes of Proteobacteria as well as Firmicutes, Acidobacteria, and the kingdom Fungi (Table 1). *Geobacter sulfurreducens* has been used as model organism to elucidate the molecular basis of electron transfer. *Geobacter sulfurreducens* produces current densities in pure culture that are among the highest of any known microorganism (Nevin, *et al.*, 2008, Yi, *et al.*, 2009). Although *G. sulfurreducens* contains genes for more than 100 c-type cytochromes (Methe, *et al.*, 2003), it has been reported that a c-type cytochrome designated OmcZ (outer-membrane cytochrome Z) is the only cytochrome that is essential for optimal current production (Nevin, *et al.*, 2009).

For application purposes, mixed cultures are more suitable for the use of complex fuels such as wastewaters, as single organisms generally metabolize quite a limited range of organic compounds. Moreover, it was shown that in a single-chamber MFC with air cathode and low internal resistance, an enriched consortium of microorganisms produced 22% more power (576 mW per m^2) than a pure culture of *Geobacter sulfurreducens* (Ishii, *et al.*, 2008).

Table 1 Microorganisms capable of current production in pure culture in microbial fuel cells.

Year	Bacteria	Higher taxonomic level	Reference
1999	*Shewanella putrefaciens* IR-1	γ-proteobacteria	(Kim, *et al.*, 1999)
2000	*Proteus vulgaris*	γ-proteobacteria	(Kim, *et al.*, 2000)
2001	*Clostridium butyricum*	Firmicutes	(Park, *et al.*, 2001)
2002	*Desulfuromonas acetoxidans*	δ-proteobacteria	(Bond, *et al.*, 2002)
	Geobacter metallireducens	δ-proteobacteria	(Bond, *et al.*, 2002)
2003	*Rhodoferax ferrireducens*	β-Proteobacteria	(Chaudhuri & Lovley, 2003)
	Aeromonas hydrophila A3	δ-proteobacteria	(Pham, *et al.*, 2003)
	Geobacter sulfurreducens	δ-proteobacteria	(Bond & Lovley, 2003)
2004	*Desulfobulubs propionicus*	δ-proteobacteria	(Holmes, *et al.*, 2004)
	Pseudomonas aeruginosa	γ-proteobacteria	(Rabaey, *et al.*, 2004)
2005	*Geothrix fermentans*	Acidobacteria	(Bond & Lovley, 2005)
	Geopsychrobacter electrodiphilus	δ-proteobacteria	(Holmes, *et al.*, 2004)
2006	*Saccharomyces cerevisiae*	Fungi	(Walker & Walker, 2006)
	Escherichia coli	γ-proteobacteria	(Zhang, *et al.*, 2006)
	Shewanella oneidensis DSP10	γ-proteobacteria	(Ringeisen, *et al.*, 2007)
	Shewanella oneidensis MR-1	γ-proteobacteria	(Bretschger, *et al.*, 2007)
2007	*Desulfitobacterium hafniense*	Firmicutes	(Milliken & May, 2007)
2008	*Acidiphilium sp.* 3.2Sup5	α-Proteobacteria,	(Borole, *et al.*, 2008)
	Ochrobactrum antrophi YZ-1	α-Proteobacteria,	(Zuo, *et al.*, 2008)
	Rhodopseudomonas palustris DX-1	α-Proteobacteria,	(Xing, *et al.*, 2008)
	Desulfovibrio desulfuricans	δ-proteobacteria	(Zhao, *et al.*, 2008)
	Therminicola sp. Strain JR	Firmicutes	(Wrighton, *et al.*, 2008)
	Hansenula anomala	Fungi	(Prasad, *et al.*, 2007)
	Klebsiella pneumoniae L17	γ-proteobacteria	(Zhang, *et al.*, 2008)
2009	*Arcobacter butzleri*	ε-proteobacteria	(Fedorovich, *et al.*, 2009)
	Bacillus subtilis	Firmicutes	(Nimje, *et al.*, 2009)
	Thermincola ferriacetica Z-0001	Firmicutes	(Marshall & May, 2009)
	Enterobacter cloacae	γ-proteobacteria	(Rezaei, *et al.*, 2009)
2010	*Corynebacterium sp. strain* MFC03	Firmicutes	(Liu, *et al.*, 2010)

Anode microbial communities in mixed culture MFCs

Microbial community analysis of MFC anode biofilms has shown that, first, there is no emergent microorganism found in all anode biofilms and second there is no typical MFC microbial community (Aelterman, 2009). On the contrary, factors like inoculum (e.g. anaerobic sludge, aerobic sludge, sediment), MFC configuration (e.g. two-chamber, single-chamber), substrate added (e.g. pure compound, wastewater) and operation conditions (e.g. temperature) determine the microbial community composition. For example, in marine sediment microbial fuel cells (SMFC) a predominance of Geobacteraceae were found mainly belonging to *Desulfuromonas* (Bond*, et al.*, 2002, Holmes*, et al.*, 2004c). In thermophilic MFCs, Firmicutes, like *Thermincola* spp., and Deferribacteres, were reported as the main bacterial groups detected on anodes (Mathis *et al.*, 2007). Chae *et al.* (Chae*, et al.*, 2009) observed a selection of different microbial communities clearly determined by the substrate used in four identical MFCs fed with four different substrates. Similarly, the concentration of the substrate used also influences the microbial composition of the anode biofilm (Phung*, et al.*, 2004, Choo*, et al.*, 2006). The effect of the inoculum used was studied in SMFCs where different communities were selected when different sediment types were used (Holmes*, et al.*, 2004c). However, due to the wide range of materials and MFC construction used in many studies (Du*, et al.*, 2007), as well as insufficient reported data, direct comparison of biofilm microbial communities is difficult. Another problem is the lack of analysis of anodes from open circuit controls, to differentiate between community members who are effective colonizers of the anode material but do not utilize the electrode as an extracellular electron acceptor and those who are specifically enriched when the anode is operated as an electron acceptor. Furthermore, when complex organic substrates serve as fuel it is expected that microorganisms that ferment these compounds to simpler substrates will also be components of the anode microbial community (Jung & Regan, 2007). As recently demonstrated in coculture studies (Ren*, et al.*, 2007) these fermentative microorganisms may have little or no capacity for electron transfer to the anode, but their metabolism is key to powering microbial fuel cells.

In several MFC systems regardless of the MFC configuration, inoculum or substrate, the predominant phyla found on the anode were Proteobacteria and

Firmicutes. Within these phyla different species were selected on the anode depending on the inoculum, substrate, MFC configuration and operation (Table 2) (Bond*, et al.*, 2002, Lee*, et al.*, 2003, Holmes*, et al.*, 2004, Kim*, et al.*, 2004, Phung*, et al.*, 2004, Choo*, et al.*, 2006, Kim*, et al.*, 2007, Liu*, et al.*, 2007, Ishii*, et al.*, 2008, Chae*, et al.*, 2009, Chung & Okabe, 2009, De Schamphelaire*, et al.*, 2010, Sun*, et al.*, 2010). Other phyla like Bacteroidetes, Deferribacteres and Actinobacteria have also been found abundant on anodes even though no strain has been shown to produce current in pure culture. The high bacterial diversity of anode microbial communities, including current producing bacteria and non-current producing microorganisms, difficult the analysis of diversity an function of anode biofilms. Fewer studies have been performed focusing on the study of Archaea in MFC anodes and no pure culture is known to produce current. Active methanogenesis has been observed in some MFC reactors (He*, et al.*, 2005, Kim*, et al.*, 2005). Ishii *et al* (2008) observed suppressed methanogenesis in a two-chamber MFC inoculated with rice field soil. However, the environmental factors influencing this competition and the mechanisms have not been elucidated.

Table 2 Anode microbial communities in MFC. The table shows the predominant taxonomic groups found in different MFC types (sediment microbial fuel cells-SMFC, two-chamber MFC and single-chamber MFC) as well as the predominant bacteria found on the anodes. Amount of 16S rRNA gene clones from predominant taxonomic groups is shown in brackets.

MFC-Type Substrate	Predominant taxonomic groups	Predominant bacteria	Reference
Sediments or soil			
SMFC Marine sediment	δ-Proteobacteria (71.3%)	*Desulfuromonas acetoxidans*	(Bond, *et al.*, 2002)
SMFC Marine sediment	δ-Proteobacteria (70 %)	*Desulfuromonas* spp.	(Holmes, *et al.*, 2004)
SMFC Thermophilic Marine sediment	Firmicutes (80%) Deferribacteres (15%)	*Thermincola carboxydiphila* *Deferribacter* sp.	(Mathis, *et al.*, 2007)
SMFC marine sediment	γ-Proteobacteria (78.9%)	Unc. γ-Proteobacteria	(Liu, *et al.*, 2007)
SMFC Saltmarsh sediment	δ-Proteobacteria (76%)	*Desulfuromonas* spp.	(Holmes, *et al.*, 2004)
SMFC Estuary sediment	δ-Proteobacteria (62.5 %)	*Desulfuromonas* spp.	(Holmes, *et al.*, 2004)
SMFC Freshwater sediment	δ-Proteobacteria (53.5 %)	*Geobacter* spp. *Pelobacter propionicus*	(Holmes, *et al.*, 2004)
Two-chamber River sediment River water	β-Proteobacteria (46.2%)	Unc. β-Proteobacteria	(Phung, *et al.*, 2004)
Two-chamber River sediment Glucose, glutamate (10)	α-Proteobacteria (64.4%)	*Aminobacter aminovorans*	(Phung, *et al.*, 2004)
Two-chamber River sediment Glutamate, glucose (200)	γ-Proteobacteria (36.5%)	*Pseudomonas aeruginosa*,	(Choo, *et al.*, 2006)
Two-chamber Rice paddy soil Cellulose	Firmicutes (39.5%) Proteobacteria (21%)	*Clostridium* spp. *Propionispora* spp. *Rhizobiales* spp.	(Ishii, *et al.*, 2008)
SMFC planted Rice root exudates Potting soil	δ-Proteobacteria (75%)	*Desulfobulbus* related *Geobacter* spp.	(De Schamphelaire, *et al.*, 2010)
Aerobic sludge			
Two-chamber Activated sludge Acetate	δ-Proteobacteria (21%)	*Geobacter sulfurreducens*	(Lee, *et al.*, 2003)
Two-chamber Activated sludge Glucose, glutamate	Bacteroidetes (29.5%) β-Proteobacteria (29.5%) γ-Proteobacteria (19.2%)	*Dysgonomonas shahii* Bacterium str. 51885 *Klebsiella oxytoca*	(Kim, *et al.*, 2006)
Two-chamber Activated sludge Chocolate WW	β-Proteobacteria (50.6%)	*Aquincola tertiarcarbonis*	(Patil, *et al.*, 2009)
Two-chamber	γ-Proteobacteria (87%)	*Aeromonas* spp.	(Chung & Okabe, 2009)

Bioreactor biomass Glucose			
Two-chamber Bioreactor biomass Effluent of MFC1	Firmicutes (64%)	*Lactococcus* sp.	(Chung & Okabe, 2009)
Two-chamber Bioreactor biomass Effluent of MFC2	Firmicutes (48%)	*Streptococcaceae* spp. *Acetobacterium* spp.	(Chung & Okabe, 2009)
Anaerobic sludge			
Two-chamber Anaerobic sludge Starch WW	β-Proteobacteria (25.0%) α-Proteobacteria (20.1%)	*Thauera* spp.	(Kim, *et al.*, 2004)
Two-chamber Anaerobic sludge Ethanol	β-Proteobacteria (83%) δ-Proteobacteria (17%)	Proteobacterium Core-1 *Azoarcus* spp. *Desulfuromonas* spp.	(Kim, *et al.*, 2007)
Two-chamber (Therm.) Anaerobic digester Acetate	Deferribacter (57.8%)	*Deferribacter* spp. *Coprothermobacter* spp.	(Jong, *et al.*, 2006)
Two-chamber Anaerobic sludge Acetate	α-Proteobacteria (48.8%) δ-Proteobacteria (31.7%)	*Thauera* spp. *Geobacter-like/* *Geobacter sulfurreducens*	(Chae, *et al.*, 2009)
Two-chamber Anaerobic sludge Propionate	Firmicutes (59.3%) β-Proteobacteria (18.5)	*Bacillus* spp.	(Chae, *et al.*, 2009)
Two-chamber Anaerobic sludge butyrate	β-Proteobacteria (59.1%) α-Proteobacteria (18.2%)	*Geobacter*-like *Geobacter sulfurreducens*	(Chae, *et al.*, 2009)
Two-chamber Anaerobic sludge Glucose	β-Proteobacteria (34%) δ-Proteobacteria (18%)	*Geobacter*-like *Geobacter sulfurreducens*	(Chae, *et al.*, 2009)
Single chamber Anaerobic sludge Glucose	Actinobacteria (34.4%) γ-Proteobacteria (37.5%)	*Nostocoida limicola* *Citrobacter freundii* *Klebsiella oxytoca*	(Sun, *et al.*, 2010)
Single chamber Anaerobic sludge Wastewater	ε-Proteobacteria (57.8%)	*Arcobacter butzleri*	(Sun, *et al.*, 2010)
Other inocula			
Two-chamber Rumen Cellulose	Firmicutes (57.5%) Deferribacteres (27.8%)	Unc. Clostridiaceae *Geovibrio ferrireductans*	(Rismani-Yazdi, *et al.*, 2007)
Single chamber MFC Biomass Inhibitor compounds	δ-Proteobacteria (50%)	Proteobacterium Core-3	(Borole, *et al.*, 2009)
Two-chamber Primary clarifier Wheat straw hydrolisate	Bacteroidetes (40%)	*Dysgonomonas wimpennyi*	(Zhang, *et al.*, 2009)
Single chamber Primary clarifier Acetate	Firmicutes (58.1%) β-Proteobacteria (25.8%)	*Clostridium* spp. *Comamonas* spp.	(Xing, *et al.*, 2010)

Mechanisms involved in the interaction between bacteria and anodes

To enable cellular respiration in a MFC and conversion of substrate to CO_2, electrons have to be transferred from central cellular metabolism extracellularly to the electrode. Currently, two mechanisms have been proposed to carry out this function; direct electron transfer and mediated electron transfer (Schröder, 2007). The electron transfer mechanisms are not exclusive and might occur in parallel.

The direct electron transfer takes place via physical contact of the bacterial cell membrane with the fuel cell anode, with no diffusional redox species being involved in the electron transfer from the cell to the electrode. The direct electron transfer requires that the microorganisms possess membrane bound electron transport proteins that transfer electrons from the inside of the bacterial cell to its outside, terminating in an outer membrane (OM) redox protein that allows the electron transfer to an anode (Schröder, 2007). Recently it has been suggested that, e.g., some *Geobacter* and *Shewanella* strains can produce electrically conducting pili (nanowires) that allow the microorganism to reach and utilize more distant solid electron acceptors (Reguera*, et al.*, 2005, Gorby, 2006). The formation of such nanowires may allow the development of thicker electroactive biofilms and thus higher anode performances. Moreover, a cytochrome that may be easily released into the biofilm matrix may also contribute to current production and it has been suggested that the *Geobacter sulfurreducens* biofilm is conductive (Reguera*, et al.*, 2006).

In mediated electron transfer the electrons are transferred from the microorganisms to electron shuttles. A diverse set of compounds can act as electron shuttles which are compounds capable of accepting electrons from one or more electron carriers within the cell and to transfer the electrons to the anode, returning to an oxidized form. Some bacteria like *Escherichia coli* (Park & Zeikus, 2000) and *Proteus* (Kim*, et al.*, 2000) need the addition of mediators to the MFC to allow the transfer of electrons to the anode, such as thionine, benzylviologen, 2,6-dichlorophenolindophenol, 2-hydroxy-1,4-naphthoquinone and various phenazines, phenothiazines, phenoxoazines, iron chelates and neutral red (Lovley, 2006). *Pseudomonas (Rabaey, et al., 2004)*, *Geothrix* (Bond & Lovley, 2005), *Shewanella* (Lanthier*, et al.*, 2008, Marsili*, et al.*, 2008) and *Lactococcus* (Freguia*, et al.*, 2009) are able to produce soluble electron shuttles to

promote electron transfer between cells and the electrode surface. Biosynthesizing an electron shuttle is energetically expensive (Mahadevan*, et al.*, 2006) and therefore an electron shuttle must be recycled many times in order to recoup this energy investment. For this reason, microorganisms that produce electron shuttles are expected to be at a competitive disadvantage in open environments in which the shuttle will rapidly be lost from the site of release (Nevin & Lovley, 2002).

1.2 The rice wetland ecosystem and methane emission

Methane is an important greenhouse gas as it can absorb infrared radiation 25 times more effectively than carbon dioxide (Schlesinger, 1997). Wetland rice was estimated to account for approximately 15% of the global methane emission (Intergovernmental Panel on Climate Change-IPCC, 2007) and its influence on the methane budget will even increase in future in correlation with the food demands of the growing human world population. The global atmospheric concentration of methane has increased from a pre-industrial value of about 715 ppb to 1732 ppb in the early 1990s, and is 1774 ppb in 2005 (IPCC, 2007).

Wetland soils are seasonally or permanently water saturated and forms the largest single source of atmospheric methane (IPCC, 2007). Rice paddies represent a unique form of wetlands characterized by the dominance of one plant species. The biogeochemistry in rice paddies is mainly controlled by the input of organic carbon and oxygen and by the availability of alternative electron acceptors such as Fe(III), nitrate, Mn(IV), and sulfate (Conrad & Frenzel, 2002). Besides soil organic matter, the organic carbon originates from decay of plant material or is released from the plant through root exudation (Hartmann*, et al.*, 2009). Oxygen is a limiting factor in flooded paddy fields. It only penetrates the first millimeters of the soil where it is rapidly consumed by respiring microorganisms (Frenzel*, et al.*, 1992). Furthermore, rice plants act as conduit for oxygen transport through the intercellular aerenchyma system thereby providing oxygen to deeper anoxic soil compartments (Grosse & Bauch, 1991, Conrad & Frenzel, 2002). As a result, three major habitats for microorganisms in paddy fields can be specified: 1) the anoxic bulk soil, 2) the oxic surface soil, and 3) the partially oxic rhizosphere with increased substrate concentration (Figure 4). In the presence of oxidants, the organic

carbon is completely oxidized to carbon dioxide. If oxygen is not available, alternative electron acceptors are reduced according to their redox potential: nitrate is thermodynamically preferred over Mn(IV), followed by Fe(III) and sulfate (Ponnamperuma, 1972). Fe(III) represents the most abundant electron acceptor in paddy fields (Yao *et al.,* 1999). Whereas oxygen and nitrate are rapidly consumed after flooding, the following Fe(III) reduction can persists for several weeks (Ponnamperuma, 1972). At the oxic-anoxic interfaces prevalent at the rhizosphere and surface soil, electron acceptors can be regenerated and a redox cycling of N, Fe, and S takes place (Fig. 4). In the absence of oxygen or alternative electron acceptors, organic carbon is disproportionated to carbon dioxide and methane. Methane is produced by methanogens as the terminal step of the anaerobic degradation of organic matter (Schutz*, et al.*, 1989, Neue, 1993). The anaerobic degradation of organic matter involves four main steps: 1) hydrolysis of polymers by hydrolytic organisms, 2) fermentation of simple organic compound by fermentative bacteria, 3) acetate formation from metabolites of fermentations by homoacetogenic or syntrophic bacteria, and 4) CH_4 formation from H_2/CO_2, acetate, simple methylated compounds or alcohols and CO_2 (Yao and Conrad, 2001). In paddy soil, acetate and H_2 are the two main intermediate precursors for CH_4 formation (Yao & Conrad, 1999). The net amount of CH_4 emitted from rice fields to the atmosphere is the balance of two opposite processes, production and oxidation by methanotrophic bacteria. Methane can be transported to the atmosphere by several mechanisms, for example via diffusion through the soil and water; however, the main fraction of methane is transported through the root aerenchyma system of the plant (Dubey, 1995).

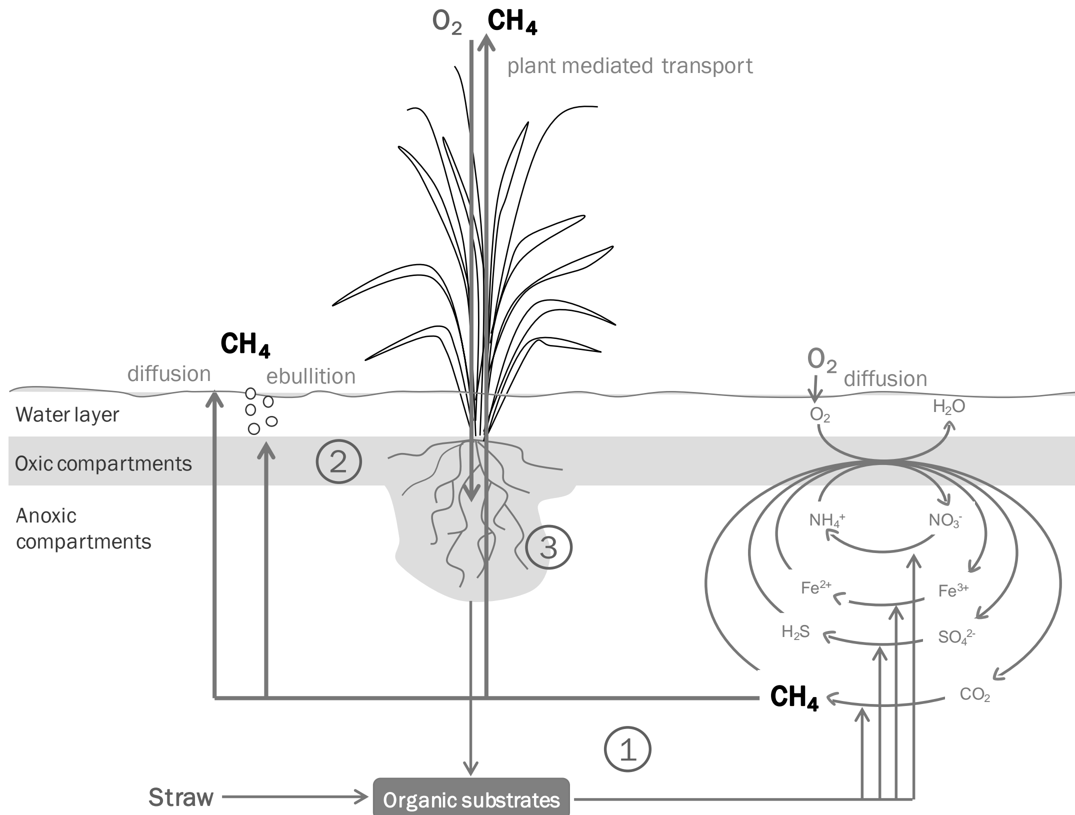

Figure 3 Scheme of the main biogeochemical processes and the habitats of the active microorganisms in a flooded rice field. 1 = anoxic bulk soil; 2 = oxic surface soil; 3 = rhizosphere. Modified from Lüke (2009) with permission.

1.3 Stable isotope probing and next generation sequencing technologies in microbial ecology

One of the biggest challenges in microbial ecology is to identify which microorganisms are carrying out a specific set of metabolic processes in the natural environment (Dumont & Murrell, 2005). Until recently, this was addressed by cultivating strains in the laboratory using defined substrates and inferring the potential roles of these and related micro organisms *in situ* in the environment. An important limitation of this method is the fact that most microorganisms cannot be cultivated under laboratory conditions (Rappe & Giovannoni, 2003). In the last decade, techniques have been developed that combine cultivation independent identification of microorganisms with metabolic analyses; examples are, the study of functional genes like *nir*S and *nir*K for denitrifiers (Braker*, et*

al., 1998) or *pmo*A for methanotrophs (Dumont & Murrell, 2005), FISH-microautoradiography (Lee*, et al.*, 1999, Ouverney & Fuhrman, 1999), and Stable Isotope Probing (SIP) (Radajewski*, et al.*, 2000). SIP involves the incorporation of stable-isotope-labeled substrates (e.g. ^{13}C) into cellular biomarkers that can be used to identify organisms assimilating the substrate. The incorporation of ^{13}C by microorganisms in a microbial community can be followed by the isotopic analysis of biomarkers like Phospholipid Fatty Acids (PLFA) (Boschker*, et al.*, 1998), DNA (Radajewski*, et al.*, 2000) and RNA (Manefield*, et al.*, 2002). This allows the identification of microorganisms that were able to use the labeled compound within a certain microbial community. The separation of ^{12}C and ^{13}C molecules is achieved by isopycnic centrifugation due to the higher mass of the ^{13}C labeled biomarker molecules. After separation of the different fractions of the centrifugation gradient, molecular analysis with T-RFLP and cloning/sequencing allows the identification of the microorganisms which incorporated ^{13}C into their biomass. DNA-SIP techniques have been used to study functionally active populations (methanotrophs, methanogens, etc.) in several environments: soils, sediments and aquifer (Dumont & Murrell, 2005, Friedrich, 2006). One of the drawbacks of DNA-SIP is the relatively long incubation times that are required for DNA replication and incorporation of the ^{13}C-label into newly synthesized DNA. Because RNA synthesis occurs at a faster rate than DNA synthesis, it is possible to obtain ^{13}C-RNA more quickly than ^{13}C-DNA. RNA-SIP has successfully been used to identify several active populations, for example; iron reducers in rice paddy soil (Hori*, et al.*, 2010), sulfate reducing toluene degraders (Bombach*, et al.*, 2010), methylotrophic bacteria in marine sediments (Moussard*, et al.*, 2009) and perchloroethene-respiring microorganisms in anoxic river sediment (Kittelmann & Friedrich, 2008). Stable isotope probing also provided valuable information on the interaction of rhizophere-microorganisms where root exudates influence the dynamics of microbial populations. One approach to study these interactions is to incubate plants with $^{13}CO_2$ and subsequently extract nucleic acids from the rhizosphere soil and analysis of the isopycnic centrifugation gradient fractions by T-RFLP or DGGE and cloning of 16S rRNA genes. This approach was applied to study the active methanogens in rhizospheric rice field soil (Lu & Conrad, 2005) and the active bacteria on rice roots (Lu*, et al.*, 2006).

Pyrosequencing, a fairly recent alternative to Sanger sequencing, involves DNA polymerase synthesizing a complementary strand of DNA in the presence of a single-stranded template and is based on the detection of pyrophosphate released during nucleotide incorporation (Ronaghi, 2001). The technology for performing pyrosequencing was developed by Margulies *et al*., (2005) and provides a new way of reducing the costs associated with sequencing. The addition of sample-specific key sequences called 'barcode' or 'tag' consisting of smaller sequences of two or four nucleotides allows the analysis of an increased number of samples at a time, reducing the cost per sample, and to perform more accurate comparative analysis of microbial communities (Roh*, et al.*, 2010). Pyrosequencing can provide between 10 and 100 times more sequencing data compared to what can be obtained using traditional cloning and sequencing (Roesch*, et al.*, 2007, Acosta-Martinez*, et al.*, 2008). Moreover, the next generation 454 Life Sciences sequencing platform, the GS FLX Titanium, provides up to 400 Mb of data per run with an average read length of 400 bp. The pyrosequencing method has been applied to study microbial communities in various ecosystems such as deep mines (Edwards*, et al.*, 2006), soils (Roesch*, et al.*, 2007, Acosta-Martinez*, et al.*, 2008, Acosta-Martinez*, et al.*, 2010), deep marine biospheres (Huber*, et al.*, 2007), chronic wounds (Dowd*, et al.*, 2008), tidal flats (Kim*, et al.*, 2008), human oral microflora (Keijser*, et al.*, 2008), wastewater microbial fuel cells (Lee*, et al.*, 2010), fermented seafood (Roh*, et al.*, 2010), rumen (Pitta*, et al.*, 2010) and potato root endophytes (Manter*, et al.*, 2010). Along with the modified pyrosequencing approaches, efficient and automated bioinformatics pipeline are needed to achieve consistent, rapid, and accurate taxonomic assignments from the 16S rRNA sequence reads (Liu*, et al.*, 2008). The advent of next-generation high-throughput sequencing techniques has created new opportunities in microbial ecosystem genomic research and discovery through cost-effective sequence throughput in a relatively short time.

1.4 Aims of this study

Bacterial diversity on anodes has been studied in the past 10 years and valuable information has been obtained aiding in the understanding of current generation from MFCs. However, many questions still need to be addressed to completely unravel the

microbiology of anodes and the effect of bacterial diversity on MFC efficiency. The recently developed SMFC fueled by root exudates is a promising system with potential application in remote wetlands or rice field soils. In marine SMFCs, *Desulfuromonas acetoxidans* is the most abundant bacterium while in freshwater SMFCs *Geobacter* spp. are often regarded as responsible for current production. The anode microbial community composition in planted SMFCs has been scarcely investigated. Moreover, the influence of anodes of SMFC on Archaea, in particular methanogens, has not been addressed.

In this PhD work, we used cultivation independent tools such as T-RFLP, cloning/sequencing of 16S rRNA, and 454-pyroseqeuncing for the study of the bacterial diversity on anodes of planted SMFC with different supports for the plant including potting soil, vermiculite and rice field soil. Potential current producers were identified by comparing anode microbial community compositions in planted SMFCs and open circuit controls. Moreover, stable isotope probing with ^{13}C-CO_2 was used to identify the anode bacteria actively degrading root exudates on anodes and potential anode reducing bacteria coupling current production with root exudate oxidation. Changes in the archaeal community composition and on methane emission were studied in unplanted SMFC with rice field soil. The following topics were addressed in order to gain more insight into bacterial and archaeal communities on anodes from SMFCs fueled by root exudates.

Chapter 3: Microbial community compositions on anodes of sediment microbial fuel cells powered by rhizodeposits of living plants

Planted SMFCs are a new type of SMFC where scarce or no data on the anode bacterial and archaeal communities are available. Anode microbial biofilms are crucial for current production and a deeper knowledge on the parameters selecting for different communities is important to improve MFC efficiencies. *Which factors affect the archaeal and bacterial community compositions on anodes of SMFC fueled by root exudates?*

Chapter 4: Identification of bacteria responsible for current production in rice soil SMFC fueled by root exudates

Rice is one of the most abundant and important agronomic products. The installment of SMFC in rice fields is one of the most promising application fields of these type of MFCs. In order to finally achieve this application it is essential to understand the microorganisms playing roles in current production of rice planted SMFC with rice field soil. *Which are the anode bacteria responsible for current production in rice field soil SMFCs fueled by rice root exudates?*

Chapter 5: Identification of bacteria coupling current production with root exudates degradation by ^{13}C-CO_2 pulse-labeling and 454-pyrosequencing

After the identification of the most abundant bacteria on the anode of rice planted SMFC with rice soil the following question remained to be answered. *Are root exudates used for current production? Which anode bacteria are actively involved in root exudate degradation?*

Chapter 6: Taming methane emissions from rice field soil with microbial fuel cells

Rice fields are a major source of methane emission, a known greenhouse gas. We showed in chapters 2 and 3 that the archaeal community changed when current was produced. *Could the introduction of an anode from a SMFC reduce methane emission from rice field soils?*

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Chapter 2

Materials and Methods

2.1 Sediment microbial fuel cells - SMFC

Rice field soil sampling and preparation

The soil used for the construction of the SMFCs was sampled from a drained rice field of the Italian Rice Research Institute “Instituto Sperimentale per la Cerealicoltura” near Vercelli (Po River valley, Italy). The soil was air dried and stored at room temperature. Before use the soil was crushed using a jaw crusher (Type BB1, Retsch, Haan) and sieved (mesh size, 5 mm).

Electrode construction

The anodes and cathodes were made of carbon felt (Alfa Aesar, Ward Hill, USA; 3,18mm thick), interwoven with graphite rods (5mm diameter, Thielmann Graphit GmbH, Grolsheim, Germany) (Fig 1, Appendix). A hole (10 mm depth by 1 mm width) was drilled into the graphite rod , a copper cable inserted into the hole, fixed using electrically conductive epoxy glue (Epo-Tek H 20 E, Polytec PT, Waldbronn, Germany) and dried at 120°C for 15 min. To insulate the connection between the graphite rod and the copper cable, insulating glue (Epo Tek H 74 F, Polytec PT, Waldbronn, Germany) was used and dried at 120°C for 10 min.

Sediment microbial fuel cells construction and operation

SMFCs were constructed to study methane mitigation (chapter 6). The SMFCs were constructed in 500 mL bottles (Schott, Mainz) using 350 g of air-dried rice field soil, which was flooded leaving a 4 cm water layer (Fig. 2, Appendix). In each SMFC, an anode was placed in the soil matrix, three pore water samplers (RHIZON, type FLEX, Soil Moisture Samplers, Eijkelkamp, Giesbeek, Niederlande) and a cathode (62 cm^2) in the overlying water layer, which was aerated with an aquarium pump. The copper cable from the anode and cathode were connected through an external resistor. Three series of SMFC (A, B and C) were constructed in duplicates (A and B) and triplicates (C) and differed in anode size, time of operation and external resistance. Series A and B were operated for 72 days and the resistance was 470 Ω until day 24 and 100 Ω from day 24 to day 72. The total anode surface (TAS) for series A was 540 cm^2 while series B had half the TAS (270 cm^2). Series C was operated for 35 days had an anode size of 270 cm^2 and an external resistance of 100 Ω. Unconnected SMFCs were constructed as controls in duplicates and triplicates exactly like the connected SMFCs. The Potential (mV) was recorded every 15 minutes with a Datalogger (Agilent 34970A, Agilent Technologies, Böblingen). Current and Power densities were calculated using Ohm's law:

$I = V / R$

Where I is the current [Ampere], V the potential [Volt] and R the resistance [Ohm].

To obtain the current density the current was divided by the total anode surface (TAS) [m^2]

$J=I/TAS$

Where J is the current density [A m^{-2}], I the current [Amperes] and TAS total anode surface [m^2].

Total anode surface of the anodes were calculated as follows:

$TAS = 2\ a\ b + 2\ d\ a + 2\ d\ a$

Where a is the length of the anode [m], b the height [m] and c the width [m] of the anode.

The power was calculated as follows:

$P = I^2 R = V^2 / R$

Where P is the power [Watts], I the current [Amperes], V the potential [Volts] and R the resistance [Ohms]

To obtain the Power density, the Power was divided by the TAS:

$P_d = P / TAS$

Where Pd is power density [W m^{-2}], P the power [W] and TAS the total anode surface [m^2].

The incubations were made in the dark and at 25°C. 5 mL pore water samples were taken in vacuumed 10 mL vials. Acetate concentration in the pore water was measured by high-pressure liquid chromatography (Krumbock & Conrad, 1991). Methane and CO_2 were measured in the vial headspace by gas chromatography (Roy*, et al.*, 1997). The ^{13}C atoms percent of CH_4 was determined by GC combustion isotope ratio mass spectrometry (Conrad*, et al.*, 2000). At the end of each experiment, anodes were sampled and stored at -80°C for further molecular studies.

Planted sediment microbial fuel cell construction and operation

Planted sediment microbial fuel cells were constructed in 2008 and 2009. Detailed information (anode and cathode size, etc) are presented in chapters 4 and 5. Each SMFCs was constructed using plastic containers which were filled with 3 kg of rice field soil (Figure 3a, Appendix). The soil was flooded with water leaving a layer of overlying water of 5 cm (Fig. 3b, Appendix). In each planted SMFC and open circuit (OC) controls two anodes were placed vertically in the soil matrix. Rice plants (*Orzya sativa* cultivar Koral) were germinated in the greenhouse on humid paper for two to three weeks. 5 days after flooding the rice field soil, three rice plants were planted in each pot and fertilizer (urea (45g/L), $Na_2HPO_4 \times 2H_2O$ (17g/L) and KCl (50g/L); 10 mL / 2Kg soil) was added twice at the beginning of the operation (days one and ten). A cathode was placed in the overlying water and the circuit was connected through a resistance (Fig. 3b Appendix). SMFCs were operated in a greenhouse facility with light:dark cycles of 12h:12h at an average temperature of 25°C (Fig 3a, Appendix). Algae and crustaceae were removed from the overlying water layer regularly. Length and number of tillers were recorded once a week. The potential (mV) was recorded every 15 minutes with a datalogger and regularly downloaded. Current and power densities were calculated as for

sediment microbial fuel cells. After operation the anodes, cathodes, soil and roots were sampled as explained below.

Harvesting and sample preparation for nucleic acid extractions

Anodes, bulk soil, cathodes and roots were sampled from the SMFCs for further molecular analysis. For this, the plant and soil were removed from the plastic container and placed on a surface previously sterilized with 70 % ethanol (v/v) (Fig 4a, Appendix). The plants were cut close to the soil surface and weighted. The cathode was removed and cut into pieces and put into a 50 mL Falcon tube and immediately stored at -80°C. To remove the anode, the plant and soil were turned 90° and the roots were cut and the soil opened until the anode was visible (Fig 4b, Appendix 1). Soil and roots were separated carefully from the anode and after the release of the anode, it was washed with sterile water several times. Anodes were cut into pieces, put in 50 mL Falcon tubes and immediately stored at -80°C. Roots were cut from different parts, washed with sterile water and stored in 10mL Falcon tubes at -80°C. Bulk soil was collected by shaking off roots and stored in 50mL Falcon tubes at -80°C.

For nucleic acid extractions samples were removed from the -80°C freezer and placed into ice. Anodes and cathodes were put into sterile Petri dishes and cut into small pieces using sterile scalpels. Root samples were mashed in a sterile mortar for nucleic acid extractions.

2.2 Stable isotope pulse labeling

For the stable isotope probing experiment, SMFCs were pulse-labeled in the light period during operation days 44-51. For this, plants from three SMFCs and two open circuit (OC) controls were covered with transparent plexiglas cylinders with a volume of 6.6 L provided with two sampling ports and a ventilator for complete gas mixing (Fig 5, Appendix). The borders of the chamber were submerged in the overlaying water and water levels inside and outside of the chamber were maintained equal by injecting N_2 or removing gas when necessary. 35mL of ^{13}C-CO_2 were added every hour, 8 times per day during 8 days (57 times in total) to the chamber by using a 50mL plastic syringe. Two

planted SMFC and one OC control served as unlabelled controls and unlabeled CO_2 was added under exactly the same conditions as the labeled setups. Samples for methane and CO_2 were taken in N_2 flushed and vacuumed 10mL vials four times per day and measured by gas chromatography as described previously (Roy *et al*., 1997). Briefly, the gas chromatograph used was an SRI 8610 C equipped with an FID detector and the carrier gas was N_2. For ^{13}C-CH_4 measurements, samples were taken in N_2 flushed and vacuumed 125mL glass bulbs twice per day, before the first CO_2 addition and after the last CO_2 addition. The samples were measured by GC combustion isotope ratio mass spectrometry by Peter Claus, Max Planck Institute, Marburg, as described previously (Conrad et al, 2000). During the night, chambers were removed in order to avoid accumulation of unlabeled CO_2. Pore water samples were taken once per day and methane, CO_2 and volatile fatty acids were measured as explained below (see soil pore water analysis).

Preparation of ^{13}C-CO_2 and unlableled CO_2 for isotope labeling

For one liter of gas, 3.4 g of ^{13}C sodium bicarbonate (sodium bicarbonate-13C 98 % atom, Campro scientific, Berlin, Germany) was filled into a 50 mL vial. After flushing with N_2 and evacuating the vial by vacuum, 8mL of phosphoric acid (50 %, v/v) was added. Immediately, the vial was connected through needles and tubing to a gas tight bag (SKC Inc, city,USA), which was filled with the produced gas. The same procedure was followed for preparation of unlabeled bicarbonate.

Methane emission rate

Methane concentration in the chamber increased linearly every day from approximately 100-200 ppmV to 800-1000 ppmV. Methane concentration in gas chamber was calculated as follows:

$m = (Mv / MW) (Cg)$

Where m is the mixing ratio gas phase [ppmV], Mv the gas volume of an ideal gas (24.78 L mol^{-1}, at 25 °C), MW the molecular weight of the gas [g/mol] and Cg the gas concentration [µg/L]

then

CH_4 (μM) = (m / Mv)

Where m is the mixing ratio gas phase [ppmV] and Mv is the gas volume of an ideal gas (24.78 L mol^{-1}, at 25 °C).

The methane emission activities were obtained from the slope of equation obtained from the graph of methane concentration [μM] vs. time [days].

Soil pore water analysis

Pore water samples were collected once per day during the labeling period into N_2 flushed and vacuumed 10mL vials from regions close to the anode by using RHIZON, type FLEX, Soil Moisture Samplers (Eijkelkamp, Giesbeek, The Netherlands). Immediately after sampling, the tubes were heavily shaken by hand and an aliquot of gas sample from the headspace was collected with a pressure lock syringe and analyzed for CH_4 using a gas chromatograph equipped with FID detector as described previously (Roy*, et al.*, 1997). ^{13}C-CH_4 was measured in a GC isotope ratio mass spectrometer by Peter Claus, Max Planck Institute, Marburg, as described previously (Conrad*, et al.*, 2000). Pore water samples were stored (-20°C) and organic acids were determined by high pressure liquid chromatography (HPLC) as described previously (Krumbock & Conrad, 1991).

The CH_4 concentration in the soil pore water was calculated as follows:

CH_4 (μM) = ((m V_{HS})/ (V_{PW} Mv))

Where m is the mixing ratio gas phase (ppmv), V_{HS} is the headspace volume [L], V_{PW} is the volume of pore water [L] and Mv is the gas volume of an ideal gas (24.78 L mol^{-1}, at 25 °C).

2.3 Molecular analysis of bacterial and archaeal communities

Nucleic acid extraction

RNA extractions (n=4) with 0.5g of anode material, bulk soil or root material were performed using a bead-beating protocol as described previously by Lueders *et al*.

(2004). The anode material was placed in a Lysing Matrix E tube (MP) with sterile zirconium beads (∅ 0,1 mm, Roth, Karlsruhe), 750 µl sodium phosphate buffer (120 mM, pH 8) and 250 µl TNS-buffer (10% sodium dodecyl sulphate, 0.5 M Tris-HCl pH 8, 0.1 M NaCl) and processed immediately in a FastPrep-24 bead-beating instrument for 45 s at 6.5 m s^{-1}. The tube was immediately transferred to ice and chilled for a minute and then centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a 2-ml Phase Lock Gel Heavy-tube (Eppendorf, Hamburg) and one volume of phenol/chloroform/isomylalcohol (PCI 25:24:1, Sigma Aldrich) was added. Phases were mixed and centrifuged for 10min at 14000 rpm. The supernatant was transferred to a new 2-mL Phase Lock Gel tube and Chloroform/Isoamylalcohol (CI 24:1, Sigma Aldrich) was added. Phases were mixed and centrifuged for 10 min at 14000 rpm. The supernatant was transferred into a 2mL tube with two volumes of polyethylene glycol (PEG 30% in 1.6M NaCl) and centrifuged for 80 min at 14000 rpm and 4°C. The supernatant was discarded and the pellet was washed with 70 % EtOH, centrifuged for 15 min at 14000 rpm and 4°C. The supernatant was discarded and the pellet was dissolved in 30-50 µl RNAse-free water or elution buffer (EB, 10 mM Tris-HCl, pH 8.5, Qiagen, Hilden) and stored at -80°C.

DNA was digested from the nucleic acid mixture by using RNAse-free DNAse. 10 µl DNAse buffer was added to 15 U of DNAse (RQ1, Promega), 60-100 µl of NA extraction completing with RNAse-free water until 400 µl. The tube was incubated at 37°C for 30min and extracted with PCI and CI as described above. The supernatant was mixed with 100% EtOH and 1/10 NaAc 3M and incubated at -20°C overnight. After centrifugation (60min at 14000 rpm and 4°C) the pellet was washed with 70% EtOH, dissolved in 30-50 µl RNAse free water and stored at -80°C.

Nucleic acids (NA) and RNA were checked by gel electrophoresis in 1% Agarose gel (SeaKem LE Agarose, Bioproducts, Rockland, Main) in TAE-buffer (40 mM Tris-Acetate, 2 mM EDTA, pH 8,5) and stained in Ethidium Bromide. NA and RNA concentrations were measured in a spectrophotometer (ND-1000, NanoDrop Technologies Inc., Wilmington, Delaware, USA) at 260nm.

Isopycnic centrifugation gradients for stable isotope probing

RNA was quantified using the Ribogreen RNA-quantification kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Extracted RNA (500ng) was density separated by isopycnic centrifugation in cesium trifluoroacetate (Amersham Biosciences, Freiburg, Germany). For this, 4.5mL of CsTFA (2.0 ± 0.05 g ml^{-1}, Amersham Bioscience, Freiburg) were mixed with 175 µL of deionized formamide and 500 ng RNA with gradient buffer (GB, 0.1 M Tris-HCl, pH 8.0; 0.1 M KCl, 1 mM EDTA) to a final volume of 750 µL. After mixing, the refraction index was measured using a refractmeter (Reichert GmbH, Seefeld) and adjusted by adding GB to a refractive index 1.3725 ± 0,0002 units. The solution was transferred into a 6-ml Polyallomer UltraCrimp-Tube (Sorvall, Kendro Laboratory Products, Newtown, USA) and centrifuged for 72hrs at 39.000rpm and 20°C in an ultracentrifuge (Sorvall Discovery 90SE, TV-865 vertical rotor, Kendro Laboratory Products, Langenselbold). The fractions were separated by using a peristaltic pump (Aladdin AL-1000, World Precision Instruments, Sarasota, Florida, USA). Using a needle (Ø 0,4 mm), DEPC water was pumped into the centrifuge tube (rate = 0.75 mL min^{-1}) displacing the content into 2 mL tubes changing tubes every 30 seconds. 14 fraction of approximately 375 µL were collected, the refraction index measured and the density of the fraction calculated.

$\rho = 257,77x^2 - 683,57x + 454,42$

where ρ is the density [g ml^{-1}] and x the refraction index of each sample.

Precipitation of the RNA in each fraction was performed by adding 1 volume of isopropanol and 1/10 volume of NaAc (3M) overnight at 4°C. After centrifugation (60 min, 14,000 rpm, 4°C) the pellet was washed with 70% EtOH, centrifuged (15 min, 14,000 rpm, 4°C) and dissolved in 30 µL of EB or RNAse free water.

PCR amplification of Reversely Transcribed 16S rRNA

16S rRNA was reversely transcribed and amplified using a single step RT-PCR system (Access Quick, Promega, Mannheim, Germany) according to the manufacturer's instructions. In a final volume of 50µL of 1 x AMV/Tfl buffer, 1mM $MgSO_4$, 100 µM from each deoxynucleotide triphosphate, 0.5 µM of each primer, 0.2 mg mL^{-1} of BSA, 4 U of AMV reverse transcriptase and 4 U of Tfl DNA-polymerase were added. The primers used were Ba27f and Ba907r for Bacteria and Ar109f and Ar912r for Archaea (Table 1). The

DNA was initially denaturated for 2 min at 94°C followed by 23 cycles of denaturation (30 s at 94 °C), annealing (30s at 52°C) and elongation (1 min, 68°C). A final elongation step (68°C for 10 min) concluded the amplification.

Table 1 Primer sequences used for T-RFLP, cloning and sequencing as well as 454-pyrosequenicng of cDNA from anode, soil samples and isopycnic centrifugation gradients.

Primer	Sequence	Target	Reference
Ba27f	AGA GTT TGA TCC TGG CTC AG	Bacteria	(Edwards, *et al.*, 1989)
Ba907r	CCG TCA ATT CCT TTR AGT TT	Bacteria	(Muyzer, *et al.*, 1995)
Ar109f	ACK GCT CAG TAA CAC GT	Archaea	(Grosskopf, *et al.*, 1998)
Ar912r	GTG CTC CCC CGC CAA TTC CTT TA	Archaea	(Lueders & Friedrich, 2002)
M13f	GTA AAA CGA CGG CCA G	Plasmid vector	Promega
M13r	CAG GAA ACA GCT ATG AC	Plasmid vector	Promega
Ba518r	GTT ACC GCG GCT GCT GG	Bacteria	(Muyzer, *et al.*, 1993)

Terminal Restriction Fragment Length Polymorphism – T-RFLP

T-RFLP analysis was performed according to Egert *et al.* (2003). PCR products were cleaned up (GenElute™PCR Clean-Up Kit, Sigma-Aldrich) and ~100 ng were digested with restriction enzymes *Msp*I or *Taq*I (Promega) for Bacteria and Archaea, respectively. Purified digest (1-2 µl) (SigmaSpin™ Post-Reaction Clean-Up Columns, Sigma-Aldrich) were mixed with 11 µl of formamide (Hidi; Applera Deutschland GmbH, Darmstadt) and 0.3 µl molecular weight marker (X-Rhodamine MapMarker® 1000, BioVentures, Murfreesboro, Tennessee, USA), and denatured for 3 minutes at 95°C. Electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). T-RFLP electropherograms were analyzed with GeneMapper® Software 4.0 (Applied Biosystems). Tables were extracted for each sample with peak size *vs.* fluorescence intensity and terminal restriction fragments (TRFs) that differed by ±1 bp in different profiles were considered as identical in order to compare T-RFLP profiles between different samples. The peak heights were standardized to the minimum sample according to Dunbar *et al.* (2000). The relative abundance of each T-RF within a given T-RFLP pattern was calculated as the peak height of the respective T-RF divided by the

total peak height of all T-RFs detected within a fragment length range between 50 and 900 bp. Cluster analysis using UPGMA algorithm and Bray-Curtis similarity index, diversity indexes (Shannon and Simpson) and principal component analysis (PCA) were performed using the PAST software (Hammer*, et al.*, 2001).

Clone libraries and phylogenetic analysis

PCR was performed as mentioned above using primers for Bacteria and Archaea (Table 1). RT-PCR products were ligated into the plasmid vector pGEM-T (Promega), and the ligation mixture was used to transform *Escherichia coli* JM109 competent cells (Promega) according to the manufacturer's instructions. White colonies were selected from LB agar ampicillin IPTG/X-Gal media and the 16S rRNA gene was amplified with vector targeting primers flanking the insert (M13F and M13R-Table 1). PCR product size was verified by gel electrophoresis and clones were sequenced bi-directionally by the Sanger method. Samples OC-A and NP-A were sequenced by Qiagen (Hilden, Germany) and sample MFC-A2 by ADIS (Max Planck Institute for plant breeding research, Cologne). Raw sequence data were processed using SeqMan software (DNAStar, Madison, USA). Clone libraries were screened for chimera by using Bellerophon (Huber*, et al.*, 2004) and Mallard software (Ashelford*, et al.*, 2006). Putative Chimera were verified by fractional treeing (Ludwig*, et al.*, 1997) and excluded from further analysis. Phylogenetic analysis was conducted using ARB 5.1 software package (Ludwig *et al.,* (2004); http://www.arb-home.de). 16S rRNA sequences from all three samples were added to the database and aligned with the Fast Aligner tool of the ARB software. Reference sequences were downloaded from the ARB Silva database (Pruesse *et al*, 2007) or National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/), added to the ARB database and phylogenetic trees were constructed by the neighbor joining method. *In silico* terminal restriction fragment sizes (*in silico* T-RFs) were obtained by searching the restriction sites of the restriction enzymes *Msp*I (C|CGG) and *Taq*I (T|CGA).

Next generation sequencing

The 16S rRNA from “heavy” and “light” gradient fractions, collected after isopycnic separation, of labeled and unlabeled SMFCs and open circuit controls were used for 454-pyrosequencing as well as rRNAs from roots and bulk soil from a labeled planted SMFC. Complete cDNA was synthesized using ImProm-II™ Reverse Transcriptase (Promega) by the following procedure: 10 ng of purified RNA and 0.5 mM of random hexamer primers were incubated at 70°C for 5 min followed by 5 min chilling on ice. The following mixture was then directly added to the tubes: 4 ml of 5x ImProm-II™ Reaction Buffer, 3 mM MgCl2, 0.5 mM of each dNTP, 20 ng of bovine serum albumin (BSA) (Roche) and 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega). The reaction tubes were incubated at 25°C for 5 min, followed by 52°C for 1 h, and were finally denatured at 70°C for 15 min. The resulting cDNA product was used directly as a template for the PCR reactions needed for pyrosequencing. Fragments of 16S rRNA genes were amplified from the cDNA, with primer sets, 27F (5’-GAGTTTGATCMTGGCTCAG) and 518R (5’-GTTACCGCGGCTGCTGG) with 10 different barcodes to sort each sample from the mixed pyrosequencing outcomes. PCR reactions were conducted in quadruplicates of 50 µl each, which were later combined to minimize reaction bias. Each PCR reaction contained 10µL of 5x Herculase II Reaction Buffer (Eurofins MWG Operon), 12.5 µM of each primer (Agilent Technologies), 1 µl of Herculase II Fusion DNA polymerase (Agilent Technologies) and 1µl of template. The following thermal program was used: 95°C for 2 min, followed by 25 cycles of 94°C for 30 sec, 55°C for 30sec and 68°C for 1 min and a single step of final elongation at 68°C for 5 min. After amplification, the quadruplicate PCR reactions were pooled and loaded on 1% agarose gel stained with ethidium bromide. PCR products were cleaned up (GenElute™PCR Clean-Up Kit, Sigma-Aldrich) and quantified by Micro-Volume UV-Vis Spectrophotometer NanoDrop (Thermo Scientific). Amplicon pyrosequencing was performed by GATC (Germany) using a 454/Roche GS-FLX Titanium instrument (Roche, NJ, USA). Equal amounts of ten samples were pooled and their sequences separated according to barcodes.

Pyrosequencing data analysis

Raw sequencing reads were quality trimmed according to published recommendations (Huse*, et al.*, 2007) using the RDP Pyrosequencing Pipeline (Cole*, et al.*, 2009) applying the following criteria: i) exact matches to primer sequences; ii) no ambiguous bases; iii) read-lengths not shorter than the 150 bp. For large scale assignments into the new Bergey's bacterial taxonomy (Garrity*, et al.*, 2004) we used the Naïve Bayesian Classifier (RDP-classifier; http://pyro.cme.msu.edu/), which provides rapid taxonomic classifications from domain to genus of both partial and full-length rRNA gene sequences along with bootstrap like confidence estimates (Wang*, et al.*, 2007). The results from the RDP classification were imported into an Excel spreadsheet and relative sequence abundance at Phylum and genus levels were compared between "heavy" and "light" fractions of the different samples and between the different SMFC compartments.

Pyrosequencing reads were aligned using Infernal probabilistic model-based aligner (Nawrocki & Eddy, 2007) obtained from the Ribosomal Database Project Group. By applying the furthest neighbour approach using the Complete Linkage Clustering application of the RDP pyrosequencing pipeline, trimmed pyrosequencing sequences could be assigned to phylotype clusters of 95% identity. Based on these clusters, rarefaction curves (Colwell & Coddington, 1994), Shannon diversities (Gotelli, 2002) and Chao1 richness estimations (Chao & Bunge, 2002) were calculated using RDP pipeline.

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Chapter 3

Microbial Community Analysis of Anodes from Sediment Microbial Fuel Cells Powered by Rhizodeposits of Living Plants

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Author contributions

LDS, NB and WV designed the study. LDS constructed and operated the sediment microbial fuel cells and performed DGGE analysis. AC performed T-RFLP analysis and cloning / sequence analysis. LD, MM and AC performed data analysis. LD wrote the manuscript and all authors contributed to the final version of the manuscript.

3.1 Abstract

Rhizodeposits of living plants can be oxidized at the anode of sediment microbial fuel cells, leading to the generation of electric current. In the present research the composition of microbial communities on the anodes of sediment microbial fuel cells fueled by rice root exudates was studied. Molecular analyses of the 16S rRNA genes (DGGE, T-RFLP and clone libraries) revealed that the electrical circuit as well as the nature of the sediment matrix (the support layer) and the presence of plants influence both the bacterial and archaeal communities associated with anodes. The data indicated that deltaproteobacterial *Desulfobulbus*-like species (56% of bacterial clones) and Geobacteraceae (16%) were highly enriched on a closed circuit anode operated in potting soil. The corresponding archaeal community was dominated by as yet uncultivated representatives of the Archaea (47% of archaeal clones) and representatives of the Methanobacteriaceae (20%), Methanosarcinaceae (18%) and Methanosaetaceae (10%).

3.2 Introduction

Living plants release substantial amounts of carbon in the soil in the form of rhizodeposits, comprising exudates, secretions, decaying cell and root material, gases and mucilage (Grayston, *et al.*, 1997). Rhizodeposits account for about 20% of carbon assimilated by photosynthesis (Gregory, 2006). They stimulate the decomposition process of native soil organic matter by indigenous microorganisms, which releases nutrients for the plant (Singh, *et al.*, 2004). Exudates, part of the rhizodeposits, furthermore act as complexing agent or ligand exchanger, valuable for nutrient provision (Hinsinger, *et al.*, 2006). Rhizodeposition substantially increases the amount of (soluble) soil organic matter (Lu, *et al.*, 2004), which is to a large extent transformed into the greenhouse gas methane in wetlands (Kimura, *et al.*, 2004). The rhizodeposits thus represent a vast flow of chemical energy into the soil matrix.

This specific flow of chemical energy can be harvested by planted sediment microbial fuel cells (SMFCs) of which the concept was recently demonstrated (De Schamphelaire, *et al.*, 2008, Kaku, *et al.*, 2008, Strik, *et al.*, 2008). A planted SMFC is a

microbial fuel cell in which the anode is continuously fed by the rhizodeposits of living plants which are thereby transformed into electricity. In its most straightforward form, a plant SMFC is an adaptation of a SMFC, which has an anodic electrode buried in a planted sediment layer, allowing microbial oxidation of reduced compounds at the anode, and a cathode in the overlying water layer, in order to close the electrical circuit.

A MFC is a unique man-made ecosystem and a significant amount of work has been performed on the microbiological aspects of the anode, to clarify the basic principles underlying the operation of the fuel cells (Choo*, et al.*, 2006, Jung & Regan, 2007, Pham*, et al.*, 2008, Chae*, et al.*, 2009). In general, MFCs contain diverse microbial communities, often dominated by Gram-negative *Bacteria* (Schaetzle*, et al.*, 2008). The nature and diversity of the microbial community present on anodes depends on several factors, being the origin of the inoculum, the nature of the substrate, the presence of a redox mediator and the redox conditions (Schaetzle*, et al.*, 2008). Compared to reactor type MFCs, the anodic communities of SMFCs are more consistent in composition. In this respect, δ-Proteobacteria appear to be a group of high importance, with a dominance of Geobacteraceae (Bond*, et al.*, 2002, Tender*, et al.*, 2002), anaerobic microorganisms known for oxidation of organic compounds with concomitant reduction of insoluble Fe(III) compounds. For many members of this family, the iron compounds turned out to be replaceable by electrodes as electron acceptors (Bond*, et al.*, 2002). With the roots and surrounding rhizosphere, the plant-MFC adds an extra parameter to the as yet multifaceted SMFC system.

In the present study, the bacterial and archaeal communities associated with anodes from rice planted SMFCs (De Schamphelaire*, et al.*, 2008) was thoroughly analyzed by molecular profiling methods as well as clone libraries. These are tools often used to describe microbial communities, taking into account also the unculturable fraction of the community. The profiling methods involved Denaturating Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP). DGGE separates PCR-amplified sequences of equal length, while T-RFLP separates sequences (fragments) with different length, both based on sequence differences. T-RFLP is very reproducible and easy to handle and can accordingly be used for the rapid (and automated) comparison of large amounts of samples in an independent way, with accurate sizing of the fragments (Moeseneder*, et al.*, 1999,

Lukow, *et al.*, 2000). DGGE is prone to biases when comparing different gels, which are limited in the number of samples that can be run on it (Moeseneder, *et al.*, 1999). Furthermore, the densiometric curves used for DGGE analysis are inferred from images, and hence highly influenced by image quality. On the other hand, T-RFLP frequently produces restriction fragments with similar or identical lengths from different species hence underestimating the diversity of samples (Avaniss-Aghajani, *et al.*, 1996). DGGE has the major advantage of sequence identification, through the excision of bands from a gel and subsequent sequencing, as will be applied here. As such, DGGE can provide in depth information about environmental samples.

The advantages of both molecular profiling techniques will be combined to evaluate the effect of several parameters, being the presence of rice plants, the support material, the operation of the electrical circuit and the anode depth on the community associated with the anode of rice planted SMFCs. Phylogenetic analysis will furthermore give insight in the composition of the anode associated biofilm, allowing to come to a first understanding of the pathways involved in the functioning of rice SMFCs.

3.3 Materials and methods

Experimental setup

Several groups of rice planted sediment microbial fuel cells (SMFCs) were set up and operated as previously described (De Schamphelaire, *et al.*, 2008). The first two groups of reactors were designated as series A. For this series, plastic containers with a total volume of 4.6 L were filled with either vermiculite (exfoliated vermiculite, Sibli SA Vermiculite et Perlite, Andenne, Belgium) or potting soil (Structural Professional type 1, M. Snebbout N.V., Kaprijke, Belgium) as supports for plant growth. The potting soil, which was based on peat enriched with 1.25 kg m^{-3} NPK (nitrogen-phosphorous-potassium) fertilizer 14-16-18, was characterized by a pH of 5-6.5, mean of 150g SO_4^{2-} m^{-3} and 20% organic substances. Vermiculite is a natural mineral which can be used as hydroponic support when exfoliated (at 900°C) (exfoliated vermiculite, Sibli SA Vermiculite et Perlite, Andenne, Belgium). Two anodic carbon felts (Alfa Aesar, 3.18 mm thickness) of 9 cm by 12 cm were placed in the support layers, at 6 and 14 cm below the support surface,

resulting in a total anodic geometric area (GA) of 216 cm2. One anode of 6 cm by 9 cm was placed at a depth of 6 cm below the surface in reactors designated for open circuit (no current harvesting was required). Connections and other components of the electrical circuit were prepared as described before (De Schamphelaire*, et al.*, 2008). The containers filled with potting soil were planted with 4 rice seedlings (*Oryza sativa* ssp. indica cultivar C101PKT), while 6 were planted in the containers filled with vermiculite. Control reactors were unplanted.

A more extensive experimental setup, designated as series B and installed in the subsequent summer as a replication in time, comprised also two groups of reactors, one filled with potting soil and one with vermiculite. In this case, plastic containers with a total volume of 3.3 L were used. Three anodic carbon felts (Sigratherm, KFA, 2.5 mm thickness), each of 6 cm by 11 cm, were placed horizontally at respectively 5, 11 and 17 cm below the support surface. This resulted in a total anodic geometric area of 198 cm^2. Cathodes were inoculated carbon felts of 5 cm by 12.5 cm (Alfa Aesar, 3.18 mm thickness). Five rice seedlings were planted per reactor, while controls remained unplanted.

Inocula were added to the anodic compartments of the above-mentioned reactors, by injecting all reactors with 10 mL of the effluent of an acetate oxidizing MFC reactor (Aelterman*, et al.*, 2006) (series A) or by applying 10 mL on each anode (series B). Soil and vermiculite matrices from series A were furthermore initially mixed with 20 mL of a methanogenic culture (presettling tank of a constructed wetland, Wontergem, Belgium).

Experimental setup – reactor operation

Apart from the type of support used to fill the reactors, there were three types of SMFC reactors. These comprise 1) reactors with plants and a closed electrical circuit, allowing the harvesting of electrical current, 2) control reactors without plants, but with closed electrical circuit 3) control reactors with plants, but with an open electrical circuit (no current generation/ electron harvesting is possible). Table 1 gives an overview of the overall reactor setup and explains the nomenclature of the reactors.

TABLE 1 Overview of reactor setups. The names of reactors refer to the type of support (S = Potting soil, V = Vermiculite), the reactor series (a = experimental seriesA, b = experimental series B), the presence (P) or absence (NP) of rice plants, the operation of the electrical circuit (CC = closed circuit, allowing electron harvesting, OC = open circuit) and at several instances a number to denote duplicate reactors. When referring to a single anode, the reactor name will be followed by the symbol H, M or L to respectively denote the high, medium and low position in the support layer.

Series	Reactor names	Support	Rice plants	Electrical circuitry	Anode depths
A	Sa-P-CC1	Potting soil	Yes	Closed	2 (H, L)
	Sa-P-CC2				
	Sa-NP-CC		No	Closed	2 (H, L)
	Sa-P-OC		Yes	Open	1 (H)
	Va-P-CC1	Vermiculite	Yes	Closed	2 (H, L)
	Va-P-CC2				
	Va-NP-CC		No	Closed	2 (H, L)
	Va-P-OC		Yes	Open	1 (H)
B	Sb-P-CC1	Potting soil	Yes	Closed	3 (H, M, L)
	Sb-P-CC2				
	Sb-P-CC3				
	Sb-NP-CC1		No	Closed	3 (H, M, L)
	Sb-NP-CC2				
	Sb-P-OC1		Yes	Open	3 (H, M, L)
	Sb-P-OC2				
	Vb-P-CC1	Vermiculite	Yes	Closed	3 (H, M, L)
	Vb-P-CC2				
	Vb-P-CC3				
	Vb-NP-CC1		No	Closed	3 (H, M, L)
	Vb-NP-CC2				
	Vb-P-OC		Yes	Open	3 (H, M, L)

Sampling and (electro) chemical analysis

Continuous potential measurements were recorded every 5 till 30 min. The processing was performed according to Logan *et al*. (2006). Negative signs were assigned to power outputs corresponding with reverse (negative) currents. All reactors were completely dismantled once all plants per series had started to senesce in order to take samples from the anodes. pH at the time of dismantlement was 6.2 ± 0.6 for potting soil supported reactors and 7.0 ± 0.5 for vermiculite supported reactors. During sampling, the carbon felt anodes were removed, cut in pieces and stored at -20°C.

DGGE and T-RFLP

For Denaturing Gradient Gel Electrophoresis (DGGE) analysis, total DNA of 2 g of anode material (wet weight) was extracted using standard methods (Boon, et al., 2000). Bacterial 16S rRNA gene fragments were amplified with the primers PRBA338fGC and P518r (Muyzer, et al., 1993) and archaeal 16S rRNA gene fragments by a nested approach (Nicol, et al., 2003), using in a first PCR round primers Ar3f and Ar9r and in a second PCR round primers Saf-GC and Parch 519r for series A, and using a non-nested approach with the primers Arc915f and 1352ar-GC (Rooney-Varga, et al., 2007) for series B. PCR products were analyzed by DGGE with a denaturing gradient ranging from 45% to 60 % for Bacteria (8% acrylamide, 16h at 38V) (Boon, et al., 2002) and 55% to 70% for Archaea (7% acrylamide, 30 min at 40 V and 16h at 70V) (Rooney-Varga, et al., 2007). Gel patterns were normalized using Bionumerics software 5.1 (Applied Maths).

For Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis, three parallel extractions with ~0.5 g of anode felt were used for DNA extraction using a bead-beating protocol as described by Lueders *et al.* (2004). T-RFLP analysis was performed according to Egert *et al.* (2003). Briefly, 16S rRNA genes were specifically amplified using primers 5' 6-carboxyfluorescein labeled (FAM) Ba27f and Ba907r for Bacteria and Ar109f and Ar912r for Archaea. Amplicons (~100 ng) were digested with the restriction enzyme *Msp*I and *Taq*I (Promega) for Bacteria and Archaea respectively. Electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Applera Deutschland GmbH, Darmstadt). T-RFLP electropherograms were analyzed with GeneScan Analysis Software 4.0 (Applied Biosystems). Only peaks ranging from 40 to 900 bp were considered. The peak heights were standardized to the minimum sample according to Dunbar *et al.* (2001).

Dendrograms for cluster analysis of both DGGE and T-RFLP profiles were based on the Pearson correlation matrix (densiometric curves) and the UPGMA algorithm (unweighted pair-group method with mathematical averages). The analysis was performed with Bionumerics software 5.1 (Applied Maths).

Clone libraries and phylogenetic analysis

16S rRNA genes from a closed circuit anode sample with potting soil and plants were specifically amplified using primers Ba27f and Ba907r for Bacteria and Ar109f and Ar912r for Archaea. PCR fragments were cloned using the pGEM-T Vector System II (Promega), and a total of 133 sequences for *Bacteria* and 52 for *Archaea* were obtained from randomly selected clones by sequence analysis at the core facility ADIS (Max Planck Institute for plant breeding research, Cologne). Clone libraries were screened for chimera by analysis with the Bellerophon server (Huber*, et al.*, 2004) and Mallard software (Ashelford*, et al.*, 2006); 44 putative chimera for Bacteria and 2 for Archaea were verified by fractional treeing (Ludwig*, et al.*, 1997) and excluded from further analysis. The diversity coverage of libraries was calculated according to the formula $C = (1 - (n_1 \times N^{-1})) \times 100$, where n_1 = number of OTUs consisting of only one species and N = number of all sequences in the 16S rRNA gene library (Wagner*, et al.*, 2002) and was 84% and 88% for *Bacteria* and *Archaea* respectively. The obtained 16S rRNA gene sequences were compared to sequences from GenBank with the BLAST server of the National Centre for Biotechnology Information (August 2009) using the BLAST algorithm (Altschul*, et al.*, 1997). Phylogenetic analysis was conducted by using the ARB software package (http://www.arb-home.de). The 16S rRNA gene sequences were added to the database, aligned with the Fast Aligner tool of the ARB software (version corrected January, 2004, released January 2005) and phylogenetic trees were constructed by the fast parsimony and neighbor joining methods. Sequences were deposited with GenBank under accession numbers GQ458057 to GQ458194.

Alternatively, bands of interest were excised from DGGE gels as described before (Aelterman*, et al.*, 2006). The final product was amplified without GC clamp, purified by means of the Qiagen PCR Purification Kit and submitted to IIT Biotech (Bielefeld, Germany) for sequencing. Sequences were manually checked using Chromas 2.33 and aligned with sequences from GenBank using BLAST (Altschul*, et al.*, 1997) (August 2009). Sequences referring to band I to V were deposited with GenBank under accession numbers GQ422145-GQ422149.

3.4 Results

Electrochemical performance of reactors

The electrochemical performances of series A and part of B were reported in De Schamphelaire *et al.* (2008) and can be found summarized in Table S1 in Supplemental Material. The first group of reactors with potting soil demonstrated that up to 2.7 times more current and up to 7 times more power could be produced in reactors with rice plants than in the reactor without plants. The first series with vermiculite - a support which does not contain organic material - demonstrated that electricity production was only possible in the presence of plants. The group of reactors with potting soil as support belonging to series B demonstrated a 3.4 times higher current output and a 9 times higher power output for reactors with plants compared to those without plants. The group reactors of series B deploying vermiculite as support presented a factor 3 higher current in the presence of plants during the second, more representative and stable experimental period (70 days).

Molecular fingerprint analysis

Clustering of the microbial communities of the initial test series (series A) revealed that the type of support had a key influence on the composition of the bacterial community (Fig. 1a and 1b). In case the inert support layer of vermiculite was used, the influence of the presence of plants (dashed arrows in absence of plants) on the bacterial community was prevalent, while no effect of the electrical circuit could be noticed. The reactors with potting soil as support - generating the highest electrical currents – demonstrated a clear shift in bacterial community between open and closed electrical circuits (full arrows for open circuit). The influence of the plants revealed an even larger shift in community according to the DGGE profiles (Fig. 1a). Yet, this was not apparent from the T-RFLP profiles (Fig. 1b). On the other hand, the bacterial T-RFLP clustering for potting soil showed a grouping according to the position of the anode. In case of the archaeal communities (Fig. 1c and 1d), the type of support had a major influence as well, while the effects of other parameters could not easily be distinguished.

A more extensive evaluation of the effects of non-support material related parameters on the microbial communities could be performed through community analysis on the anodes of the extended reactor setup of series B. When considering the bacterial anodic communities from the reactors with vermiculite (Fig. 2a and 2b), a distinction between the different reactor units could be made, regardless of the position of the anodes. DGGE profiles from the anodes in absence of plants showed an obvious cluster. The effect of the electrical circuit was not apparent from the profiles. Archaeal communities from vermiculite series B (Fig. 2c and 2d) were also largely clustered in the absence of plants. It was however impossible to distinguish an effect of the electrical circuit or the anode depth. Overall, the archaeal clustering patterns from both DGGE and T-RFLP analysis demonstrated a high similarity.

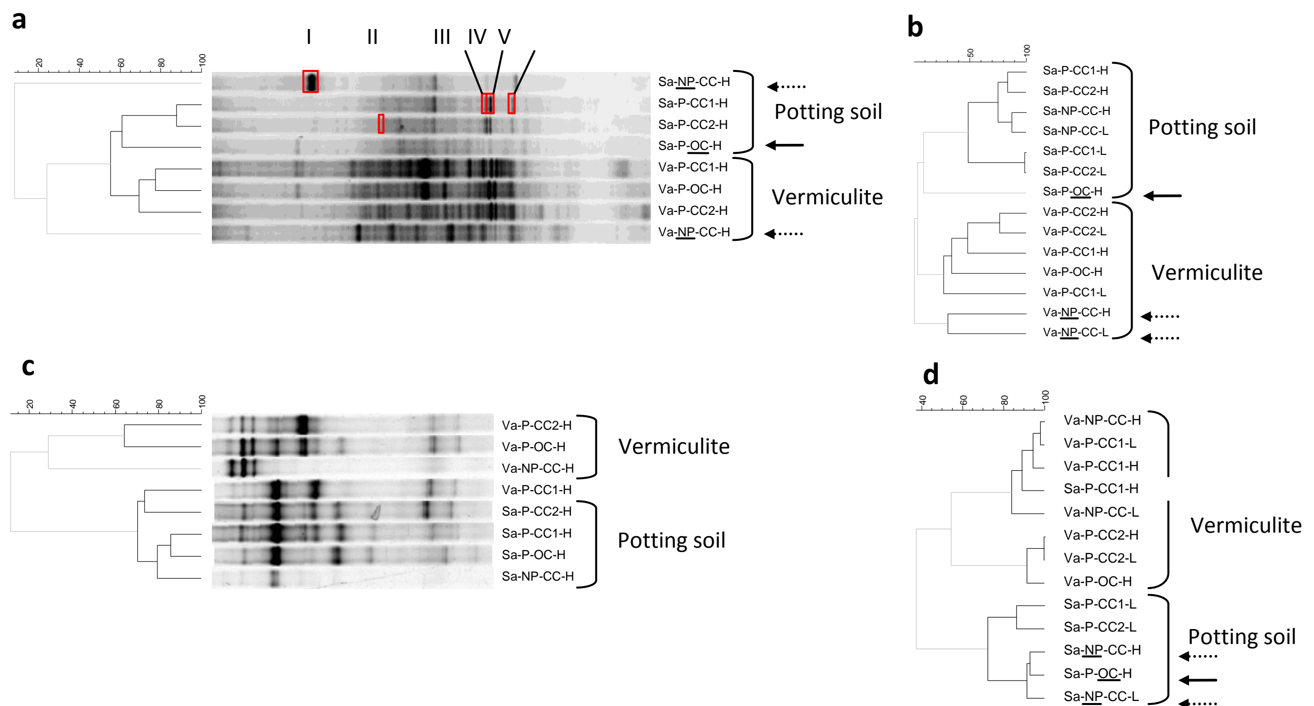


Figure 1 Clustering of bacterial and archaeal 16S rRNA gene profiles of anodes of reactor series A with vermiculite and potting soil as support material. a) Bacterial DGGE profiles b) Bacterial T-RFLP profiles c) Archaeal DGGE profiles d) Archaeal T-RFLP profiles. Clustering is performed according to Pearson's correlation matrix and the UPGMA algorithm. Dashed branches refer to cluster cutoff as calculated through Bionumerics based on Point-Bisectional Correlation. Significant effects of the absence of plants are marked by dashed arrows, while open circuits are marked by full arrows. Excised DGGE bands I to V are marked.

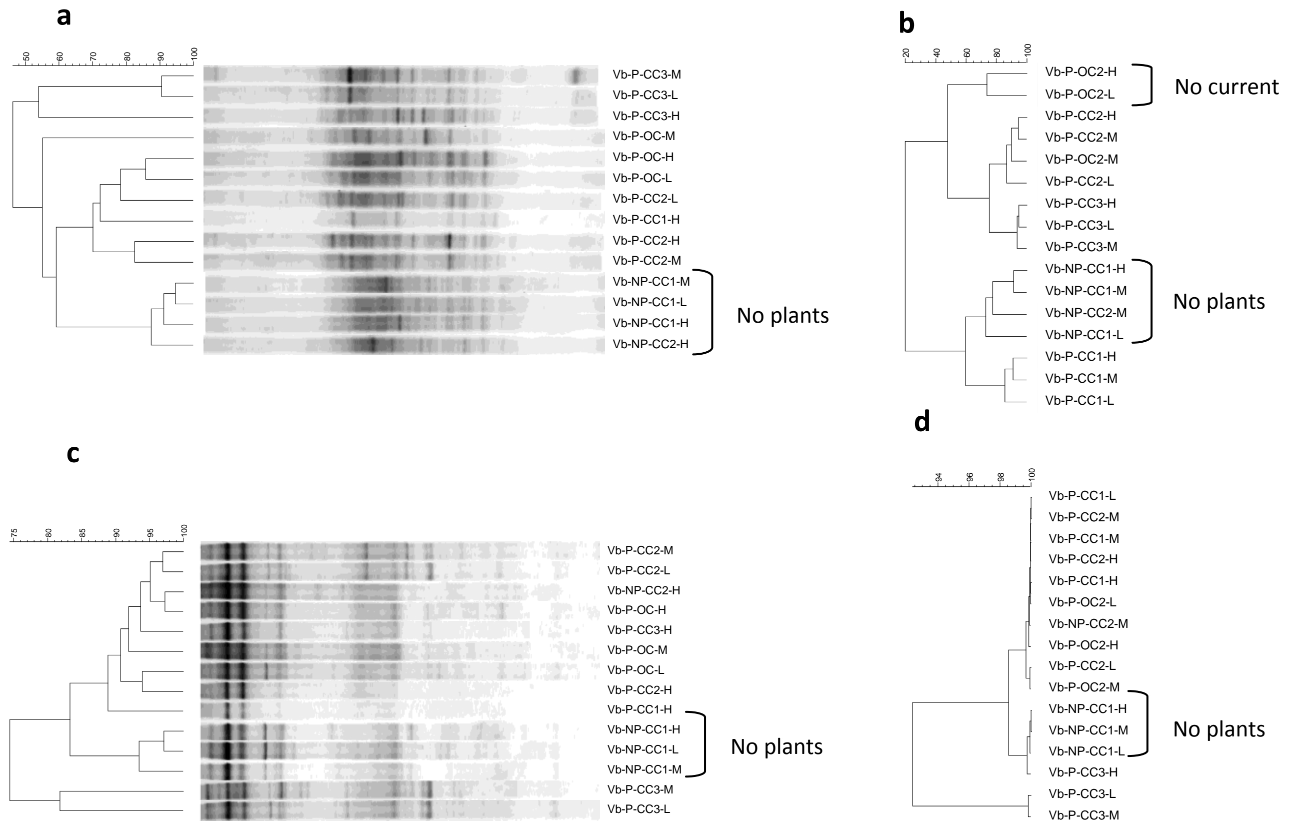


Figure 2 Clustering of bacterial and archaeal 16S rRNA gene profiles of anodes of reactor series B with vermiculite as support material. a) Bacterial DGGE profiles b) Bacterial T-RFLP profiles c) Archaeal DGGE profiles d) Archaeal T-RFLP profiles. Clustering is performed according to Pearson's correlation matrix and the UPGMA algorithm.

T-RFLP performed on potting soil series B could make a distinction between different reactor operation strategies, related to electrical circuit as well as to plant presence (Fig.3b). The differences between reactor operations could not be equally visualized through DGGE (Fig. 3a), although a separate cluster was formed by the samples of the most productive non-planted SMFC. No grouping according to anode position could be observed. When observing the archaeal community profiles (Fig. 3c and 3d), a grouping of the open circuit reactors could be noticed. The effect of plants could to some extent be observed in DGGE profiles but was absent from T-RFLP profiles.

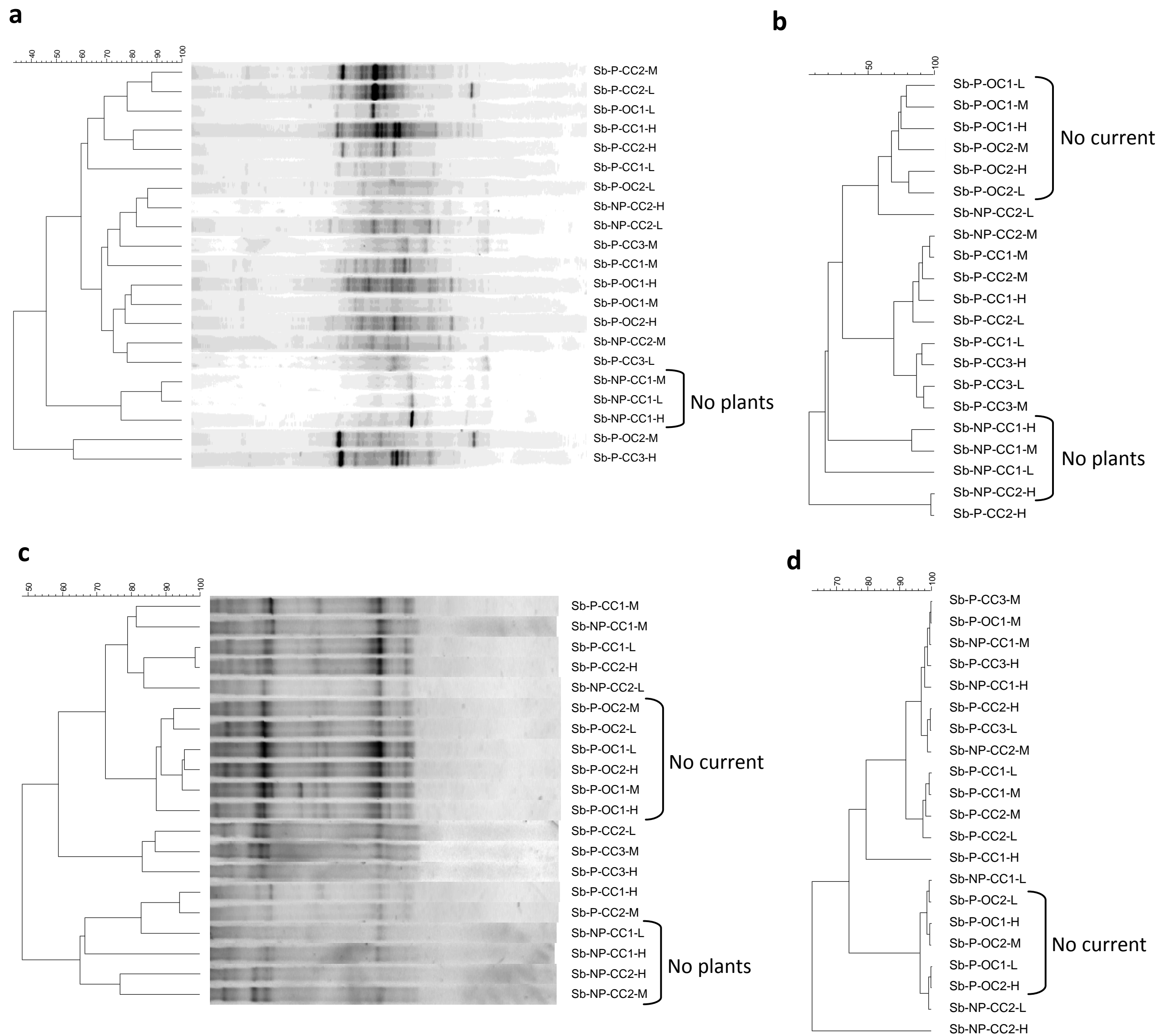


Figure 3 Clustering of bacterial and archaeal 16S rRNA gene profiles of anodes of reactor series B with potting soil as support material. a) Bacterial DGGE profiles b) Bacterial T-RFLP profiles c) Archaeal DGGE profiles d) Archaeal T-RFLP profiles. Clustering is performed according to Pearson's correlation matrix and the UPGMA algorithm.

Phylogenetic analysis

Clone libraries were made for the bacterial and archaeal communities residing on the anode of a current producing rice planted SMFC with potting soil as support and are represented by the phylogenetic trees in Figure 4. Figure 5 represents the relative

abundance of the most important phylogenetic groups found on current and non-current producing anodes from planted and non-planted rice-SMFCs based on the clone libraries and T-RFLP patterns.

According to clone libraries and PCR product abundance as evaluated by T-RFLP analyses (Fig. 4a, 5a), the most common bacterial groups on the closed circuit anode with plants were those of the *Desulfobulbus* cluster (56% of all clones) and Geobacteraceae (16% of all clones). δ-Proteobacteria made up a total of 75% of all bacterial clones. Other affiliations of importance were Chlorobi (8%), Chloroflexi (6%) and Bacteroidetes (3%). Based on the assignment of T-RFs and the relative abundances of peaks in the T-RFLP fingerprints of current and non-current producing anodes from soil supported SMFCs with and without rice plants (Fig. 5a), the different operation of the electrical circuit resulted in a prominently different community composition. The non-current producing anode namely was dominated by uncultured Bacteroidetes. In the absence of plants, more phylogenetic groups could be detected on a current-producing anode.

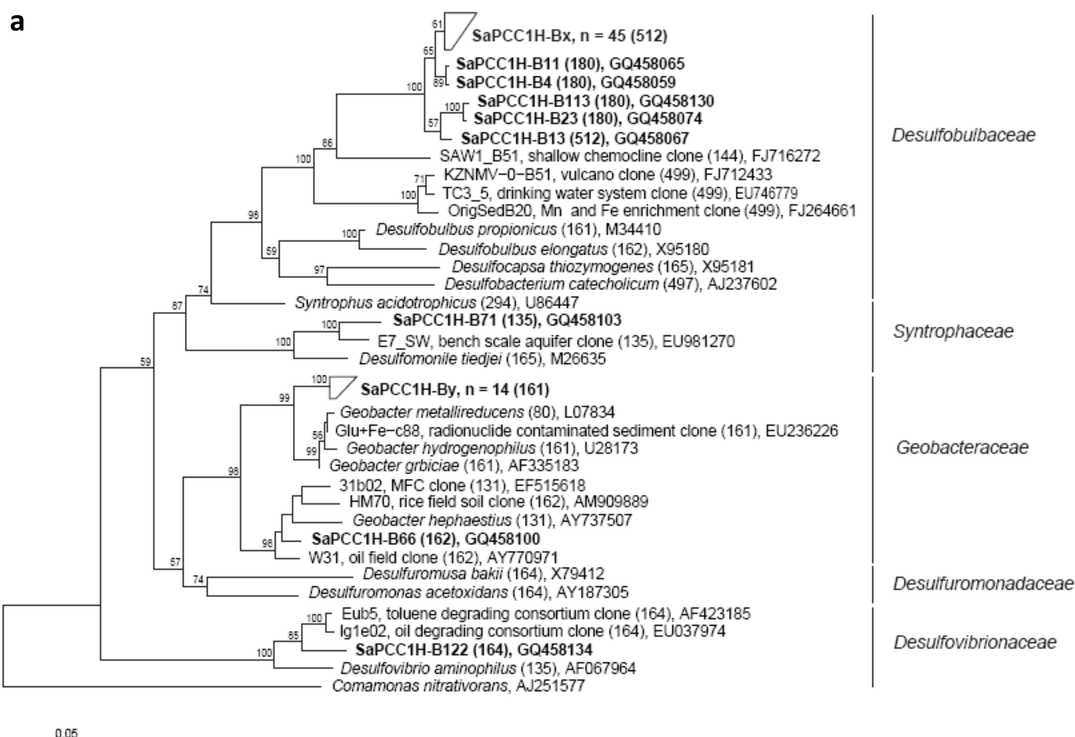
Through the excision of DGGE-bands, the enrichment of Geobacteraceae and *Desulfobulbus* on current producing anodes could also be shown: bands III, IV and V (Fig. 1), which were more abundant on current harvesting than on non-current harvesting anodes, showed the highest similarity with respectively *Geobacter metallireducens* GS-15 (accession number CP000148, sequence identity 96%), *Geobacter hydrogenophilus* strain H2 (NR025974, (Coates, *et al.*, 1996), 88%) and an uncultured *Desulfobulbaceae* bacterium clone (EF613400, (Kleinsteuber, *et al.*, 2008), 92%). Band III and IV showed 92 respectively 96.5% sequence identity with the *Geobacter* related clone sequences while band III showed 90% identity with the *Desulfobulbus* related clones. Band II was 94% identical with uncultured *Sulfurovum* sp. (GQ243133 and GQ242284) and other uncultured ε-Proteobacteria. Band I, excised from a reactor without plants, was 100% identical with an uncultured *Nitrospira* sp. (EU043588, (Tarlera, *et al.*, 2008)) and an uncultured *Nitrospira*-like bacterial clone from a rice paddy soil (EF613810).

The clones from the archaeal clone library (Fig. 4b, 5b) could be assigned to a limited number of cultured archaeal groups, with the most dominating families being Methanobacteriaceae (20% of all clones), Methanosarcinaceae (18%) and Methanosaetaceae (10%). A large group of clone sequences (47%) however was most

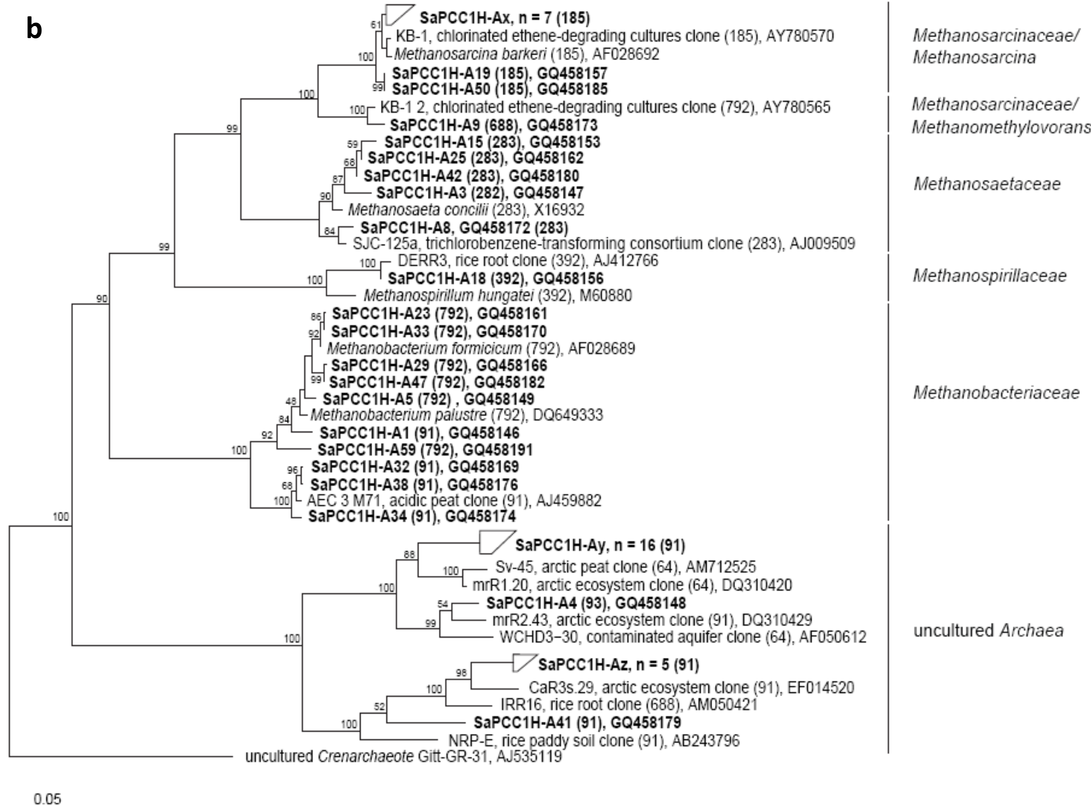
closely affiliated with uncultured Archaea. These clone sequences clustered forming two groups within the uncultured Archaea, one being the single largest clone group (35%) found in the clone library. None of the clones were closely related to any of the novel lineages termed rice clusters I to VI (RC-I to RC-VI) (Lueders & Friedrich, 2000). When comparing T-RFLP fingerprints for current and non-current producing anodes, a shift in archaeal community compositions could be derived (Fig. 5b). The production of current led to a strong increase in the relative abundance of the groups most closely related with uncultured Archaea (fourfold increase), Methanobacterium (fourfold increase) and a strong decrease in the relative abundance of Methanosaetaceae (fourfold decrease). The archaeal composition on the current producing non-planted anode could be regarded as intermediate.

Figure 4 Phylogenetic trees of a) δ-Proteobacteria 16S rRNA gene sequences and b) Archaeal 16S rDNA sequences from clones retrieved from the upper anode of a SMFC planted with rice, with potting soil as anodic support layer and operated with a closed electrical circuit (series A – Sa-P-CC1-H). The trees were constructed through the neighbor-joining method with Jukes Cantor correction. The bar indicates 5% sequence divergence. Bootstrap values higher than 50% (for 1000 iterations) are shown at the nodes of the trees. Numbers in brackets represent the *in silico* T-RF in base pairs.

a



b



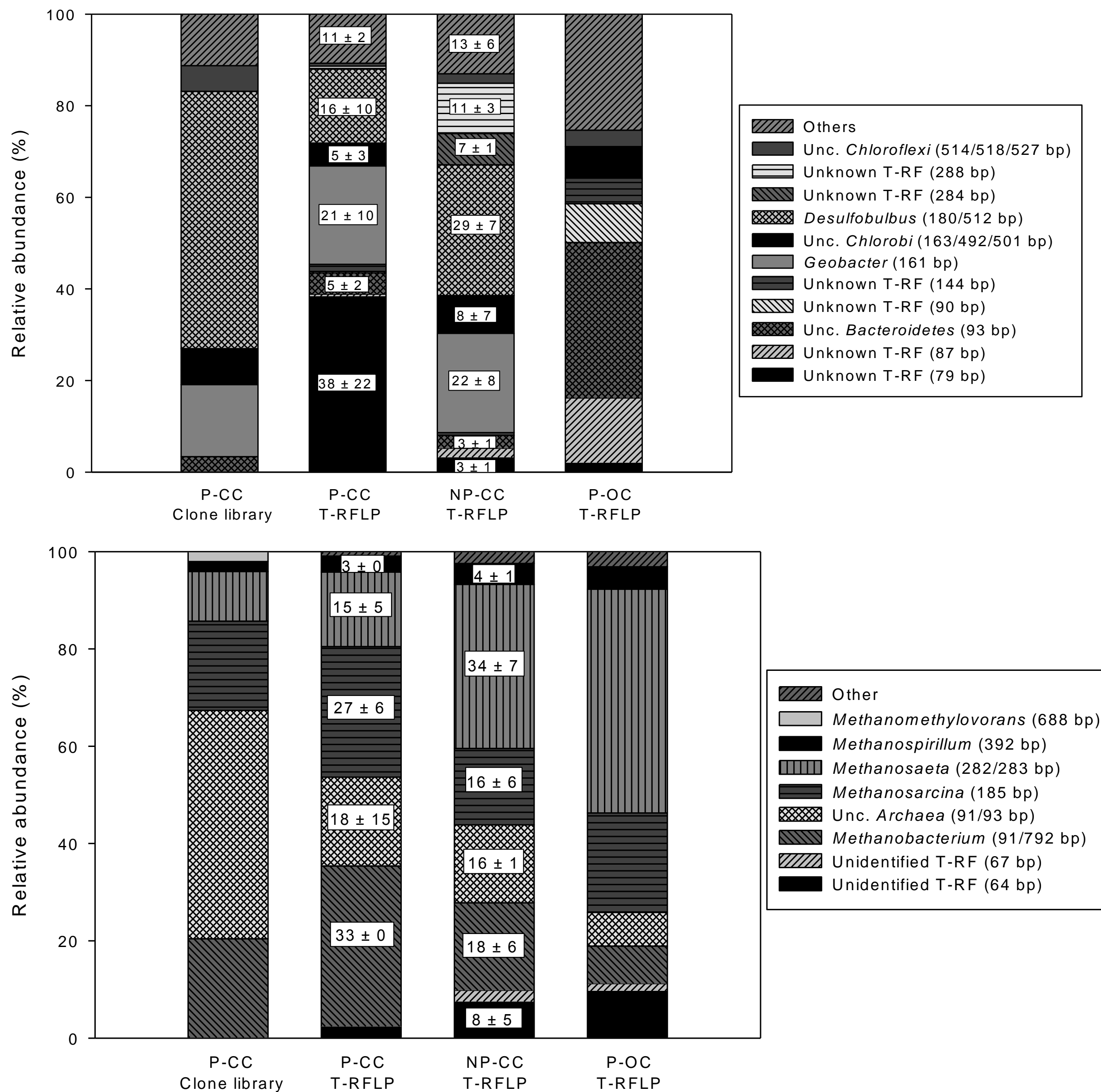


Figure 5 Comparison of relative abundance of phylogenetic groups on anodes in closed circuit (CC) and open circuit (OC) with (P) and without (NP) rice plants. a) Bacteria and b) Archaea. The analyzed samples originated from potting soil series A (Sa-P-CC1-H, Sa-NP-CC-H and Sa-P-OC-H). The phylogenetic affiliations given are the closest relatives. For the T-RFLP profiles, these are obtained through comparison with the *in silico* T-RFs from the corresponding clone library. “Others” contains all groups with an abundance of < 3% in case of Bacteria, comprising unc. OD1, *Sphingobacteria*, *Desulfomonile*, (unc.) *Spirochaeta,* unc. δ-Proteobacteria, *Desulfovibrio*, unc. Planctomycetes, unc. OP11 and unknown T-RFs and < 2 % in case of Archaea, comprising unknown T-RFs. Unc. = Uncultured. Unknown T-RF (fragment length in bp) = affiliation of the fragment could not be deduced.

3.5 Discussion

Community shifts - Effect of support

The type of support matrix had a prevailing influence, as demonstrated in Figure 1. Although the reactors with both types of support received the same inoculum mixture in each series, the potting soil itself was more colonized than the exfoliated vermiculite. Hence the anodes in vermiculite were likely to be more influenced by the added inoculum. The great difference in composition - organic matter content and mineral nutrient status – as well as differences in pH, texture and physical structure further affected the residing microbial communities (Marschner*, et al.*, 2001, Certini*, et al.*, 2004).

Community shifts - Effect of rice plants

The presence of plants was a factor of major importance. This was especially apparent for the bacterial communities found in reactors with vermiculite as support (Fig. 1a, 1b, 2a and 2b), where the plants were the only source of organic compounds, but was to a large extent also applicable for reactors with potting soil as support (Fig. 1a, 1b, 3a and 3b). The presence of plants, releasing a range of organic compounds, considerably stimulates the growth of soil micro-organisms (Hinsinger*, et al.*, 2006). Moreover, several studies suggest that plants select for taxonomic and functional groups in the rhizosphere (Singh*, et al.*, 2004). The effect of plants was also observable with archaeal communities, but was less pronounced than with Bacteria.

Community shifts - Effect of electrical circuit

The microbial community on anodes is considered to be responsible for the generation of electrical current (Rabaey & Verstraete, 2005) and hence fulfils a pivotal role in MFCs. The closing of the electrical circuit, allowing a capture of electrons by the anodes, resulted in a shift in the bacterial community, a phenomenon which has also been observed in conventional non-planted SMFCs (Bond*, et al.*, 2002, Holmes*, et al.*, 2004).

For both series, this shift was clear in case of potting soil as support (Fig. 1a, 1b and 3b) but not in case of vermiculite. The low current output – and hence low level of electron harvesting by the anode - of vermiculite series B, of which the plants were degenerated at the time of dismantlement, could be a partial reason for this. Notably, samples from reactor Vb-P-CC1, which produced a negative current near the time of sampling, clustered distinct (T-RFLP) (Fig. 2b) or with samples from an open circuit reactor (DGGE) (Fig. 2a). Also for potting soil series B, the 'erroneous' clustering of reactor Sb-NP-CC2 (Fig. 3b) could be based on the level of electrical current, which was 3 to 6 times less than that of the other reactors in closed circuit. It hence seems that not only the occurrence of electron capture but also the level of electron capture is a determining factor for the bacterial community.

The research regarding archaeal anodic communities is largely unexplored so far. Ishii et al. (2008) found more (methanogenic) Euryarchaeota in chamber MFCs inoculated with rice paddy field soil in open circuit than in closed circuit. Also in the present research it could be demonstrated that the electrical circuit had an effect on the Archaea, although Archaea were less influenced by the electrical circuit than Bacteria. The effect was only apparent when potting soil was used as a support (Fig. 1d, 3c and 3d).

Community shifts - Effect of anode depth

Reimers *et al.* (2006) found that the diversity of bacterial communities increased with anode depth along a vertical graphite rod. In the present research, the effect of anode depth was minor. This is most likely related to the interruption of the typical redox gradient due to the dense root systems, unequally releasing oxygen and organic substrates into the support matrix (Brune*, et al.*, 2000).

Phylogenetic community analysis

The results from the bacterial clone library (Fig. 5a) and the excised DGGE bands led to the same conclusions, being that Desulfobulbaceae and Geobacteraceae were dominant on current harvesting anodes. Furthermore, these groups were absent (not detected, T-RFLP) and/or of much lower importance (DGGE) on the corresponding open circuit

anode. δ-Proteobacteria and more specifically Geobacteraceae have often been found enriched on closed circuit anodes (Bond*, et al.*, 2002, Holmes*, et al.*, 2004, Jung & Regan, 2007). As mentioned before, Geobacteraceae respire organic compounds such as acetate with concomitant reduction of insoluble Fe(III) compounds and often a solid electrode (Tender*, et al.*, 2002). *Desulfobulbus* (and/or *Desulfocapsa*) species have also been found enriched on anodes: for instance in setups with marine and salt-marsh sediments (Holmes*, et al.*, 2004) and an ocean cold seep (Reimers*, et al.*, 2006). The sulfate reducing *Desulfobulbus propionicus* has been found able to oxidize organic compounds (but not acetate) with electrode reduction (Holmes*, et al.*, 2004). On the other hand, due to the importance of acetate as an intermediate in sediments and the occurrence of S^0 precipitates on anodes, the role of *Desulfobulbus* on anodes has been suggested to be linked to their ability to anaerobically oxidize S^0 to sulfate with the electrode as electron acceptor (Holmes*, et al.*, 2004) and/or their ability to disproportionate S^0 to sulfate and sulfide (Ryckelynck*, et al.*, 2005). Hereby *Desulfobulbus* would be able to recycle sulfate as an electron acceptor (Ryckelynck*, et al.*, 2005, De Schamphelaire*, et al.*, 2008): Organic compounds could be oxidized up till acetate during dissimilatory sulfate reduction by *Desulfobulbus* and/or other sulfate reducers, while acetate could for instance be oxidized by sulfate reducers other than *Desulfobulbus* (Widdel & Pfennig, 1977) or Geobacteraceae. It should be mentioned that sulfate reduction can take place at a certain distance from the anode. Sulfide, as the product from sulfate reduction, is assumed to be (abiotically) oxidized to S^0 at the anode and could then be returned to sulfate by *Desulfobulbus*. The Desulfobulbaceae found enriched in the present rice planted SMFC system could be involved in similar processes, i.e. direct current generation from organic substrates, indirect (sulfur mediated) oxidation of plant substrates or yet to be unraveled mechanisms. The sulfate content of the peat-derived potting soil (mean of 150 mg SO_4^{2-} L^{-1}, Snebbout N.V., pers. comm.) is not restrictive for any of the options. The *Desulfobulbus* related sequences found in this work were only 89% similar with *Desulfobulbus propionicus* and might hence represent a new species.

There was a significant presence of *Chloroflexi* in the clone library, but their relevance for a closed circuit anode was not clear when comparing T-RFLP profiles. *Chloroflexi* have been found enriched on the anode of a cellulose-fed MFC inoculated

with rice paddy field soil (Ishii*, et al.*, 2008). The bacterial species found in the present research do not correspond with those found important in an earlier research regarding rice planted SMFCs (*Natronocella*, *Beijerinckiaceae*, *Rhizobiales*), employing paddy field soil (Kaku*, et al.*, 2008), probably due to different initial soil microbial community compositions. Bacteroidetes, known to be proficient in degrading biopolymers such as chitin and cellulose (Kirchman, 2002), were enriched on the open circuit anode. A *Nitrospira* – related species proved significantly present in the absence of plants. *Nitrospira* is known for its obligately chemolithotrophic growth by oxidation of nitrite to nitrate (Noll*, et al.*, 2005). Its presence could be related to the lower consumption of nutrients from the NPK-fertilized potting soil.

Almost half of the sequences derived from archaeal clones from the closed circuit anode (47%) were most closely related to uncultured Archaea, and not to any of the known methanogenic lineages (Erkel*, et al.*, 2005) or novel rice cluster lineages (Grosskopf*, et al.*, 1998, Lueders & Friedrich, 2000), including the methanogenic RC-I (Erkel*, et al.*, 2005), which is found in paddy fields as well as peat bogs (Basiliko*, et al.*, 2003). This could indicate that a major part of the *Archaea* present on a closed circuit anode was not methanogenic. The archaeal sequences (Fig. 5b) which could be assigned originated from a few important groups, and although the sample did not originate from a genuine rice paddy soil, these groups were comparable with those found dominant in a rice paddy soil (Grosskopf*, et al.*, 1998, Weber*, et al.*, 2001). All these assigned sequences originated from methanogenic Archaea (Garcia, 1990). The results however indicate that the current production might have influenced the methanogenic activity and/or pathway.

Upon closing the electrical circuit, there was a decrease in the total relative abundance of known methanogens and an intriguing enrichment with uncultured (non-methanogenic) Archaea*.* These observations indicate that methanogenesis might have been suppressed in closed circuit conditions and/or that anode related processes directly or indirectly promoted growth of a certain group of Archaea. When referring to each methanogenic group individually, there was a decrease in the relative abundance of the strictly acetotrophic *Methanosaeta*, while there was an increase in the relative abundance of *Methanobacterium* spp. (CH_4 production from H_2 and CO_2 and/or formate). There was only a minor increase (from 20% to 23%) in the relative abundance of the

generalist *Methanosarcina* spp. (CH_4 production from H_2 and CO_2, acetate and/or methyl compounds). These changes might reflect an increased importance of hydrogenotrophic methanogenesis compared to acetotrophic methanogenesis. The rationale for this might be the readily oxidizable nature of acetate as a substrate for MFCs – the plant SMFC reactors were even inoculated with an acetate adapted anode inoculum (Aelterman*, et al.*, 2006) – resulting in decreased acetate levels near the surface of a closed circuit anode. Anode respiring Bacteria have also been found to oxidize H_2 at an anode, this at a much lower (factor 70) (Torres*, et al.*, 2007) to equal (Bond & Lovley, 2003) rate as that of anodic acetate oxidation. However, the observed increase in the relative abundance of *Methanobacterium* on a closed circuit anode suggests that methanogenic substrates other than acetate - as remaining from the anodic processes - gained in significance. The intermediate archaeal composition on a current producing but non-planted anode could be related to a lower availability of acetate as anode substrate in the absence of plants.

In rice paddy fields acetate contributes about 65 to 80% to methane production (Chin*, et al.*, 1999). By removing the most important substrate for methane production in paddy fields and providing a competing electron acceptor through the anode, it could be inferred that the overall methane production rate can decrease, as was the case with the addition of ferrihydrite as alternative electron acceptor (Lueders & Friedrich, 2002). This hypothesis is consistent with the phylogenetic results but can however not with certainty be inferred.

Comparison of profiling methods

Similarity dendrograms were inferred through two different methods of molecular profiling. Although the results did not always correspond completely, the overall results obtained through DGGE and T-RFLP profiles were analogous. Similar findings have been reported before (Moeseneder*, et al.*, 1999, Nunan*, et al.*, 2005, Smalla*, et al.*, 2007, Szekely*, et al.*, 2009).

The variability that could be observed can be explained by the intrinsic methodological differences arising from the two different techniques, the use of different primer sets and biases, such as from PCR amplification. Olsen and Woese (1993) also

stated that some level of disagreement should be expected when comparing molecular phylogenies, owing to intrinsic random errors in every inferred tree. In the present research, some effects, for instance the influence of the support or the influence of the plant in vermiculite series, were evident from both molecular analyses. Other effects were only evident from one of the two analyses and/or were not entirely consistent for all samples. This shows that the techniques can be complementary and allows discerning between the weight of the influencing factors. Several researches report T-RFLP to have the highest resolution (Moeseneder*, et al.*, 1999, Nunan*, et al.*, 2005, Szekely*, et al.*, 2009), while others report DGGE to have the highest discriminative power (Enwall & Hallin, 2009). In the current research, T-RFLP and DGGE had about an equal contribution in the discernment of factors of influence.

3.6 Conclusions

The bacterial and archaeal communities residing on anodes from planted SMFCs were explored through various molecular techniques. The type of support applied in the model systems had a major effect on the resulting communities. In reactors with vermiculite support, the presence of plants, as the only source of organic compounds, was a major factor of influence, especially for the bacterial communities. In reactors with potting soil, both the influence of the rice plants and the electrical circuit was apparent. It could be demonstrated that not only the bacterial but also the archaeal communities on sedimentary anodes were significantly altered upon electron harvesting. In the present case, this appeared to be related to an enrichment with uncultured (possibly non-methanogenic) Archaea. Additionally, it seemed that hydrogenotrophic populations gained importance while acetotrophic populations lost importance, implying acetate to be an important anodic substrate. Closed circuit anodes in potting soil were enriched with *Desulfobulbus*-related species and Geobacteraceae.

3.7 References

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3.8 Supplementary data

Table S1 Electrochemical performance of sediment microbial fuel cells with potting soil and vermiculite. Current and power densities are shown for potting soil reactors (Series A and B) and vermiculite (Series B) TAS stands for total anode surface. More data can be found in de Schamphelaire *et al*., 2008.

Parameter	Potting soil (Series A)	Unplanted control	Potting soil (Series B)	Unplanted control	Vermiculite (Series A)	Unplanted control
Current density (mA m^{-2} TAS)	56 ± 9	21 ± 4	32 ± 9	9 ± 6	21 ± 4	-0.3 ± 0.3
Power density (mW m^{-2} TAS)	12 ± 3	1.7 ± 0.8	4.6 ± 2.8	0.5 ± 0.5	10 ± 4	-0.004 ± 0.006

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Chapter 4

Rice Root Exudates Select for Novel Electrogenic *Geobacter* and *Anaeromyxobacter* Populations on Sediment Microbial Fuel Cell Anodes

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Author contributions

AC and MWF designed the study. AC and LD constructed and operated sediment microbial fuel cells. AC performed all sample analysis and data analysis. AC and MWF wrote the manuscript. All authors contributed to the final version of the manuscript.

4.1 Abstract

A novel type of sediment microbial fuel cell (SMFC) allows coupling the oxidation of rice root exudates to current production. We analyzed the composition of the microbial community on anodes from rice planted SMFCs with rice field soil by terminal restriction fragment length polymorphism (T-RFLP) and cloning/sequencing of 16S rRNA. In closed circuit planted SMFCs, clones related to δ-Proteobacteria and Chloroflexi were highly abundant (49% and 21%, respectively) and predominant sequences were related to *Geobacter* and *Anaeromyxobacter* populations (19% and 15%, respectively) as well as Anaerolineae (17%). In open circuit control anodes, not allowing the transfer of electrons to the anode, δ-Proteobacteria (32%), β-Proteobacteria (20%), Chloroflexi (12%), α-Proteobacteria (10%) and Firmicutes (10%) predominated. Moreover, specific clone clusters within the phylogenetic radiations of the genera *Geobacter* (92-95% sequence identity) and *Anaeromyxobacter* (90-95% sequence identity) were stimulated the most in SMFCs fueled by root exudates as revealed by comparison to an unplanted control. The presence of an electron accepting anode had a strong influence also on methanogenic Archaea. Hydrogenotrophic methanogens such as Methanobacteriales and Methanosarcinales were more abundant on closed circuit anodes (21%) than on open circuit control anodes (10%), whereas acetoclastic Methanosaetales were more abundant on open circuit control anodes (31%) compared with SMFCs (9%). Our study showed that electron accepting anodes and rice root exudates selected for distinct microbial populations of potential anode reducers and suppressed acetoclastic methanogens.

4.2 Introduction

Microbial fuel cells (MFCs) are bioelectrochemical devices for green energy production, in which current is produced by microorganisms capable of converting chemical energy present in organic matter directly to electric energy (Logan, et al., 2006, Lovley, 2006, Davis & Higson, 2007). In MFCs, certain microorganisms are capable of using the anode as terminal electron acceptor of their respiratory chain (Lovley, 2008). A number of bacterial strains are capable of electrical current production belonging to the five classes

of Proteobacteria, as well as the phyla Firmicutes and Acidobacteria, and up to now, at least 29 anode reducing bacterial strains are known (reviewed in Logan (2009); for recently described electrode reducing microorganisms refer to Fedorovich *et al.* (2009), Marshall and May (2009), Nimje *et al.* (2009), Rezaei *et al.* (2009), Xing *et al*. (2009), Liu *et al.* (2010)). Microbial community analysis of MFC anode biofilms has shown that, (1) there is no emergent microorganism found in all anode biofilms and (2) there is no typical MFC microbial community (Aelterman, 2009). One strategy to determine which microorganisms contribute to power production is to identify those microorganisms that selectively colonize anode surfaces (Jung & Regan, 2007). Several phylogenetic groups have been found to be predominant in different types of MFCs, mainly determined by the inoculum used (Holmes, *et al*., 2004), the substrate used for feeding (Jung & Regan, 2007, Chae*, et al.*, 2009, Chung & Okabe, 2009, Sun*, et al.*, 2010) and the anode material (Liu*, et al.*, 2007). For example, bacteria belonging to the family Geobacteraceae were found dominant on anodes from marine sediment microbial fuel cells (SMFC) (Bond*, et al.*, 2002, Tender*, et al.*, 2002, Holmes*, et al.*, 2004) as well as on anodes from a MFC initiated with anaerobic digester sludge inoculum fed with acetate, glucose or lactate (Jung & Regan, 2007). In contrast, Liu *et al*. (2007) reported the predominance of γ-proteobacteria in marine SMFCs. Other recent studies differing in inocula (e.g. activated sludge, anaerobic digester sludge, rice field soil, rumen), energy sources (e.g. synthetic wastewater, ethanol, methanol, cellulose, acetate, butyrate, propionate, glucose) and/or MFC configurations (two chamber MFC, single chamber MFC, sediment MFC) reported the predominance of γ-Proteobacteria (Kim*, et al.*, 2006), β-Proteobacteria (Kim*, et al.*, 2007, Chae*, et al.*, 2009), α-Proteobacteria (Ishii*, et al.*, 2008, Ishii*, et al.*, 2008) or Firmicutes (Rismani-Yazdi*, et al.*, 2007, Chung & Okabe, 2009) on anodes.

Recently, a sediment type microbial fuel cell was employed to produce electrical current from rhizodeposits of rice, e.g. organic exudates from roots, and thus, ultimately using photosynthetically fixed carbon (De Schamphelaire*, et al.*, 2008, Kaku*, et al.*, 2008). Previously, we detected a high abundance of *Desulfobulbus* related spp. and *Geobacter* spp. in 16S rRNA gene clone libraries from anodes of planted SMFC with potting soil (De Schamphelaire*, et al.*, 2010). In similar systems but with rice field soil, the natural support for rice plants, the bacterial community on anodes was analyzed by

fingerprinting using DGGE, but the composition of the bacterial community on anodes was not comprehensively determined (Kaku*, et al.*, 2008). Much less is known about the involvement of Archaea in MFCs. Archaea have been found on anodes of two-chambered with a predominance of methanogens (Ishii*, et al.*, 2008, Chung & Okabe, 2009). Chung & Okabe (2009) also reported that methanogens were located near or on the anode surface in a two-chamber MFC. In planted SMFC with potting soil not only methanogens were detected but a group of uncultured Euryarchaea was enriched on the anode (De Schamphelaire*, et al.*, 2010). So far, Archaea have not been shown to produce electrical current in pure culture in an MFC system.

Carbon felt anodes immersed in rice field soil can be colonized by microorganisms and used as alternative electron acceptor by anode reducing bacteria producing electrical energy in so called SMFCs (Holmes*, et al.*, 2004, De Schamphelaire*, et al.*, 2008). The addition of a rice plant into the system increases of the input of organic matter for anode reduction, thereby increasing current outputs of SMFCs. Here, we studied the active bacterial and archaeal community compositions in rice planted SMFCs, unplanted SMFCs and non-current SMFCs with rice field soil as support for the plant by terminal restriction fragment length polymorphism (T-RFLP) and cloning/sequencing of the 16S rRNA. By comparing the different microbial community compositions on anodes we were able to determine the main current producing bacteria in planted SMFC with rice field soil, the natural support for rice plants.

4.3 Materials and Methods

Microbial fuel cells

Three series (A, B and C) of planted SMFCs were constructed and operated including open circuit and unplanted controls. All sediment MFCs were constructed using rice field soil as support for rice plants. Soil was sampled in 2006 from a drained rice field of the Italian Rice Research Institute “Instituto Sperimentale per la Cerealicoltura” near Vercelli (Po River valley, Italy). Soil parameters were as described previously (Chin & Conrad, 1995). The soil was air dried, sieved (mesh size 5mm) and stored at room temperature as described previously (Chin & Conrad, 1995).

MFC series A and B were operated for 104 and 90 days in 2007 and 2008, respectively in the greenhouse in the Max Planck Institute, Marburg. All series included two planted SMFCs (MFC-A1, MFC-A2; MFC-B1, MFC-B2) one open circuit control for each series (OC-A and OC-B) and for series A, also an unplanted control (NP-A). Plastic containers were filled with 3 kg of rice field soil, which were flooded with water leaving a 5 cm layer of overlying water. In each planted SMFC and controls two anodes of 10 cm by 10 cm were placed vertically in the soil matrix giving a total anode surface (TAS) of 425 cm^2 and a cathode of 10 cm by 10 cm was placed in the overlying water layer. Carbon felt anodes and cathodes (Alfa Aesar, Ward Hill, USA; 3,18 mm thick) were attached to an insulated cable with a graphite rod (5 mm diameter, Thielmann Graphit GmbH, Grolsheim, Germany). Closed electrical circuits had an external resistance of 470 Ω for series A and 470 Ω and 100 Ω (470 Ω until day 45 and 100 Ω from day 45 until the end of the experiment) for series B. Three one week old plants (*Orzya sativa* cultivar Koral) were planted in each pot (except for the unplanted control). Fertilizer (5 mL / kg soil; urea [45g/L], Na_2HPO_4 x $2H_2O$ [17g/L] and KCl [50g/L]) was added twice during the first weeks. Series A and B were operated in a greenhouse with light:dark cycles of 12h:12h at an average temperature of 25 °C. The electric potential (mV) was recorded every 15 minutes with a datalogger (Agilent 34970A, Agilent Technologies, Böblingen) and current and power densities were calculated as reported previously (Logan*, et al.*, 2006). At the end of the incubation, anodes and bulk soil were sampled and stored at -80°C for further molecular analysis.

Series C was operated from November 2007 till March 2008 in a greenhouse at LabMET (Ghent University) and consisted of a planted SMFC (MFC-C), an unplanted control (NP-C), a planted open circuit control (OC-C) and an unplanted open circuit control (OCNP-C). The construction and operation was similar as for series A and B with the following differences: 1.2 kg of rice field soil were used per SMFC, two carbon felt anodes were placed horizontally with a total anode surface of 231 cm^2 and SMFCs were operated at an average temperature of 28°C with light:dark cycles of 16h:8h each planted with five three week old rice seedlings (*Oryza sativa* cultivar C101PKT).

Molecular analysis of bacterial and archaeal communities

Nucleic acid extraction, PCR and T-RFLP

RNA extractions (n=4) with 0.5g of anode material or bulk soil each were performed using a bead-beating protocol as described previously (Lueders *et al*. (2004)). T-RFLP analysis was performed according to Egert *et al.* (2003). Briefly, 16S rRNA was reversely transcribed and PCR amplified using a single step RT-PCR system (Access Quick, Promega, Mannheim, Germany). 5' 6-carboxyfluorescein labeled (FAM) primers were used to specifically amplify Bacteria (FAM-Ba27f and Ba907r) and Archaea (Ar109f and FAM-Ar912r). PCR products were cleaned up (GenElute PCR Clean-Up Kit, Sigma-Aldrich) and ~100 ng were digested with restriction enzymes *Msp*I or *Taq*I (Promega) for Bacteria and Archaea, respectively. Purified digest (1-2 µl) (SigmaSpin Post-Reaction Clean-Up Colums, Sigma-Aldrich) were mixed with 11 µl of formamide (Hidi; Applera Deutschland GmbH, Darmstadt) and 0.3 µl molecular weight marker (X-Rhodamine MapMarker 1000, BioVentures, Murfreesboro, Tennessee, USA), and denatured for 3 minutes at 95°C. Electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Applied biosystems). T-RFLP electropherograms were analyzed with GeneMapper Software 4.0 (Applied Biosystems). Tables were extracted for each sample with peak size *vs.* fluorescence intensity and terminal restriction fragments (TRFs) that differed by ±1 bp in different profiles were considered as identical in order to compare T-RFLP profiles between different samples. The peak heights were standardized to the minimum sample according to Dunbar *et al.* (2000). The relative abundance of each T-RF within a given T-RFLP pattern was calculated as the peak height of the respective T-RF divided by the total peak height of all T-RFs detected within a fragment length range between 50 and 900 bp. Cluster analysis using UPGMA algorithm and Bray-Curtis similarity index, diversity indexes (Shannon and Simpson) and principal component analysis (PCA) were performed using the PAST software (Hammer, *et al.*, 2001). As discrepancies between *in silico* and observed T-RF sizes might occur (Schutte, *et al.*, 2008) we further refer to the *in silico* T-RF value.

Clone libraries and phylogenetic analysis

16S rRNA transcripts from MFC-A2 were cloned and sequenced using specific primers. In addition, bacterial clone libraries were also constructed for control anode samples (OC-A and NP-A). RT-PCR was performed as mentioned above however using unlabeled primers for Bacteria and Archaea. RT-PCR products were ligated into the plasmid vector pGEM-T (Promega), and the ligation mixture was used to transform *Escherichia coli* JM109 competent cells (Promega) according to the manufacturer's instructions. White colonies were selected from LB agar ampicillin IPTG/X-Gal media and the 16S rRNA gene was amplified with vector targeting primers flanking the insert (M13F and M13R). PCR product size was verified by gel electrophoresis and clones were sequenced bidirectionally by the Sanger method. Samples OC-A and NP-A were sequenced by Qiagen (Hilden, Germany) and sample MFC-A2 by ADIS (Max Planck Institute for plant breeding research, Cologne). Raw sequence data were processed using SeqMan software (DNAStar). Clone libraries were screened for chimera by using Bellerophon (Huber, *et al.*, 2004) and Mallard software (Ashelford, *et al.*, 2006). Putative chimera were verified by fractional treeing (Ludwig, *et al.*, 1997) and excluded from further analysis. Phylogenetic analysis was conducted using ARB 5.1 software package (Ludwig, *et al.*, 2004); http://www.arb-home.de). 16S rRNA sequences from all three samples were added to the database and aligned with the Fast Aligner tool of the ARB software. Reference sequences were downloaded from the ARB Silva database or GenBank (National Center for Biotechnology Information-NCBI, http://www.ncbi.nlm.nih.gov/), added to the ARB database and phylogenetic trees were constructed by the neighbor joining method. *In silico* terminal restriction fragment sizes (*in silico* T-RFs) were obtained by searching the restriction sites of the restriction enzymes *Msp*I (C|CGG) and *Taq*I (T|CGA).

4.4 Results

Electrochemical performance

The planted SMFC reactors with rice field soil were operated for approximately three months. During this time, all planted SMFC attained higher electrical currents than the unplanted controls (Fig. S1). Until day 40 current production was low, both in planted and unplanted SMFCs of series A and B (MFC-A, 7 ± 1 mA m^{-2} TAS; MFC-B, 8 ± 1 mA m^{-2} TAS; NP-A, 5 ± 2 mA m^{-2} TAS). From day 41 onwards current increased by a factor of two in planted SMFCs (MFC-A, 15 ± 1 mA m^{-2} TAS, MFC-B, 18 ± 1 mA m^{-2} TAS) while the unplanted SMFC remained constant (NP-A, 8 ± 1 mA m^{-2} TAS) (Fig. S1). Maximum power density averages were up to three times higher for planted SMFC (2.8 ± 0.3 for MFC-A, 1.6 ± 0.2 mW m^{-2} for MFC-B) than for unplanted controls (0.9 mW m^{-2} for NP-A), and open circuit potentials attained up to 900 mV. For series C, closed circuit potentials of planted SMFCs remained nearly constant, while that of unplanted SMFC decreased through time. Maximum average current of planted SMFCs was 4 times higher than that of unplanted controls (8 ± 1 mA m^{-2} TAS, versus 2 ± 2 mA $m^{-2)}$ while the maximum power average was around 7 times higher (0.6 ± 0.2 mW m^{-2} TAS versus 0.09 ± 0.05 mW m^{-2} TAS), and open circuit potentials attained up to 900 mV.

Microbial community analysis

Differences in bacterial and archaeal community compositions on anodes and in bulk soil from planted SMFC (series A and B) and controls (unplanted and open circuit), were assessed by T-RFLP and cloning/sequencing of the 16S rRNA at days 104 and 90 respectively.

Bacterial community composition

T-RFLP analysis revealed that microbial community compositions differed in planted SMFCs and open circuit (OC) controls. The main difference detected was the increase of the relative abundance of T-RF 159-bp in planted SMFCs compared to OC controls (Fig. 1a). A 124-bp T-RF was the second most abundant T-RF on the planted SMFCs and the

most abundant T-RF on OC controls. A 148-bp T-RF was twice as abundant on OC samples as in planted SMFCs. The relative abundance of other T-RFs present on OC samples also decreased in the planted SMFC (T-RFs of 511-bp, 489-bp, 436-bp, 148-bp, 138-bp) indicating a stimulation of few populations on current producing anodes. This was reflected in lower Shannon and Simpson diversity indices which were 2.626 and 0.8142, respectively for the planted SMFC and 3.173 and 0.8822, respectively for the OC control. Principal component analysis showed that planted SMFC samples formed a separate cluster and most of the variance of principal components 1 and 2 were explained by the differences in relative abundance of 124-bp and 159-bp T-RFs (Fig. 2). The T-RFLP data indicated that the bacterial community on anodes from planted SMFC have a lower diversity and evenness than the OC control with high predominance of T-RFs 124-bp and 159-bp.

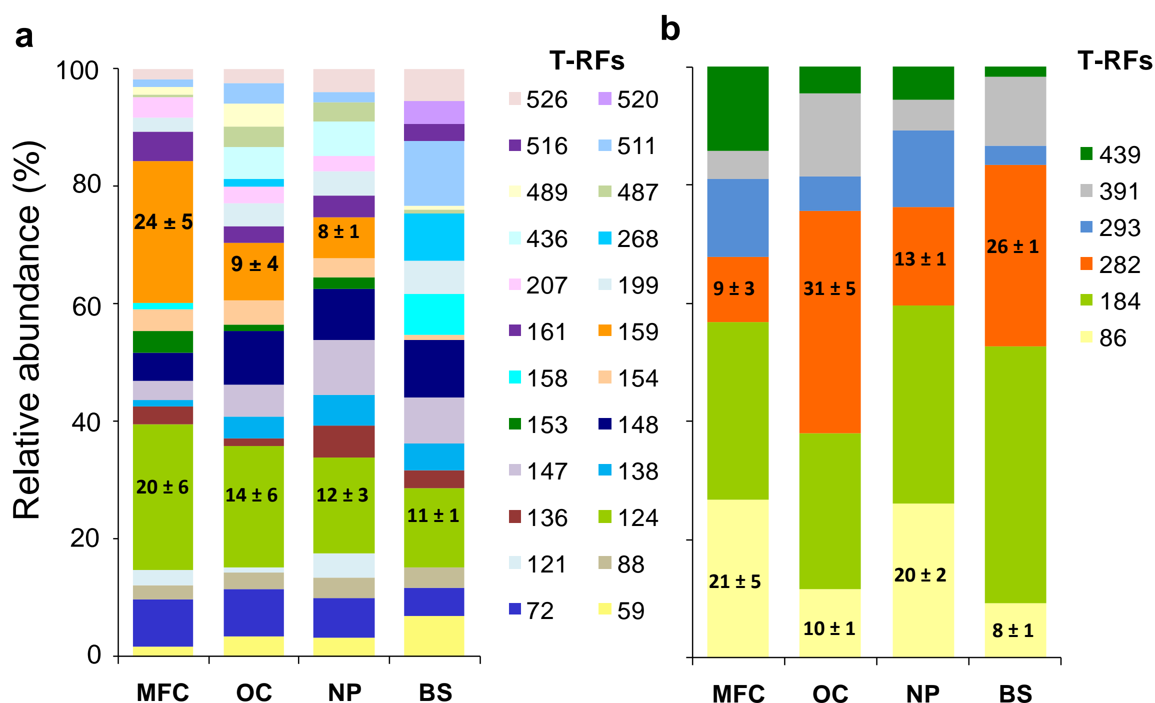


Figure 1 T-RFLP analysis of bacterial (a) and archaeal (b) 16S RNA on anodes from planted sediment MFC (MFC), unplanted sediment MFC (NP), open circuit control (OC) and bulk soil samples (BS). Shown are T-RF relative abundance averages (%) for each sample analyzed (MFC: n=4, NP: n=2, OC: n= 3, BS: n=4). T-RFs with less than 2 % and 5 % relative abundance for Bacteria and Archaea respectively were not included in the graphic representation. On the left of each graph the sizes of the T-RFs are shown in base pairs. Relative abundances of T-RFs 159/161-bp (*Geobacter* spp.), 124-bp (*Anaeromyxobacter* spp.), 86-bp (Methanobacteriales) and 282-bp (*Methanosaeta* spp.) were added to the figure with the corresponding standard deviation.

Bacterial compositions of the unplanted SMFC and bulk soil samples differed strongly and some T-RFs present in bulk soil samples were not detected in the unplanted SMFC (e.g. T-RFs 158-bp and 268-bp) (Fig. 1a). In contrast, T-RF 159-bp was not present in bulk soil samples, however, abundant in the unplanted SMFC. T-RF 124-bp was abundant in all samples analyzed (Fig. 1a). Cluster analysis showed that bulk soil samples formed a cluster with an intragroup similarity of 85% and that the unplanted control was more similar to open circuit control samples (Fig. S2a).

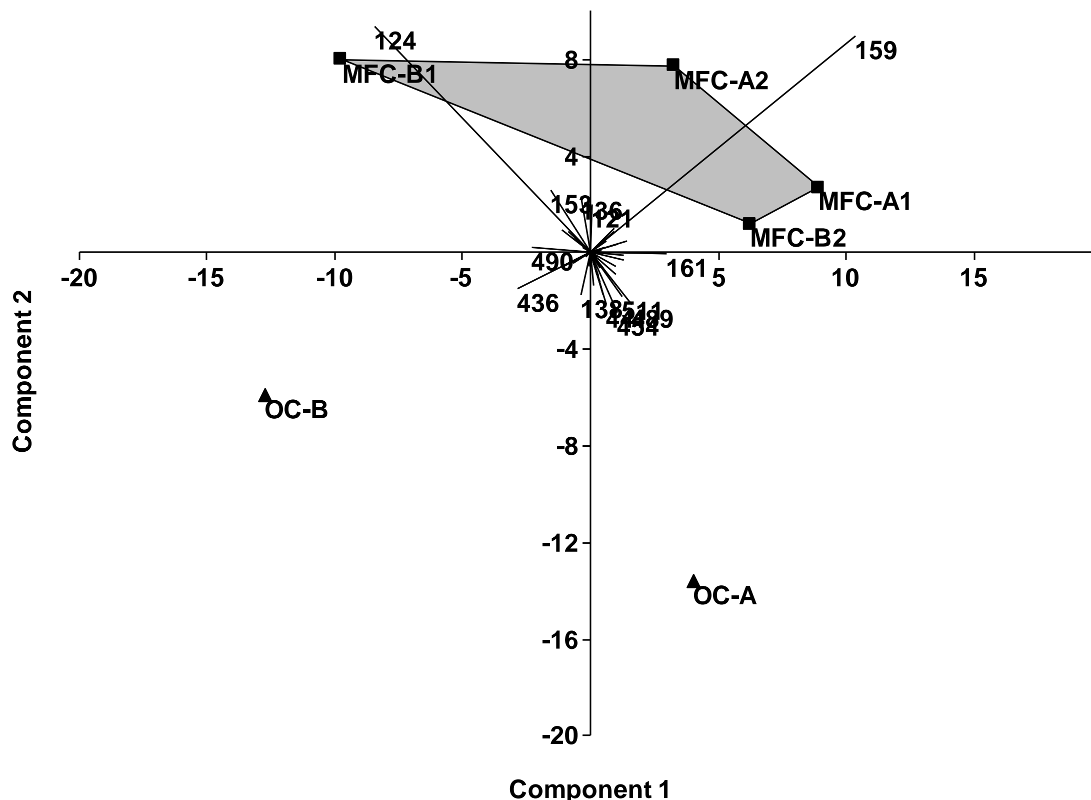


Figure 2 Principal component analysis (PCA) of bacterial T-RFLP profiles from planted SMFC anode samples (filled squares) and open circuit control anode samples (filled triangles). The clustering reflects differences in the T-RFLP profiles. The vectors shown indicate the T-RFs which explain the clustering of the samples and only values for main T-RFs are shown. The smallest convex polygon containing all planted SMFC samples is shown in grey. Components 1 and 2 explain 87.6 % of the variance

In order to get more insight into the differences in composition of the anode samples and to be able to assign the T-RFs found in the T-RFLP analysis three clone

libraries from anodes of the planted SMFC (MFC-A2), unplanted control (NP-A) and open circuit control (OC-A) were analyzed. By *in silico* restriction of 16S rRNA clone sequences the identification of main T-RFs found in the T-RFLP analysis was possible and their relative abundance was in general in accordance with clone abundances found in the clone libraries (Table S1).

Cloning and sequence analysis of 16S rRNA revealed that the bacterial communities were different in each of the anode samples. On planted SMFC anode samples clones related to δ-Proteobacteria (49%) and Chloroflexi (21%) were predominant while in the open circuit (OC) control sample, δ-Proteobacteria (32%) and β-Proteobacteria (20%) followed by Chloroflexi (12%), Firmicutes (10%) and α-Proteobacteria (10%) were most prominent (Table 1). The unplanted (NP) control sample was dominated by clones related to δ-Proteobacteria (31%) and Chloroflexi (24%) followed by α-Proteobacteria (11%) and β-Proteobacteria (11%) (Table 1). Within the δ-Proteobacteria, most of the clones from all anode samples were related to the family Geobacteraceae and the order Myxococcales but different sequence patterns were found (Fig. 3a and 3b, Table 1). Clones related to the Geobacteraceae were predominant in SMFCs, both planted (19%) and unplanted (16%), compared to the OC control (5%), indicating a stimulation of *Geobacter* related populations in current producing systems. *Geobacter*-related clones fell into three main clusters (Fig. 3a) all, which had an *in silico* T-RF of 161/163 bp. Twice as many cluster 1 clone sequences were detected in the planted SMFC (11%) compared to the unplanted control (6%) indicating a stimulation of cluster 1 *Geobacter* populations by rice root exudates (Fig. 3a). Cluster 1 clone sequences were closely related to clone sequences from rice field soil (98-100% sequence identity) however, *Geobacter chapellei* (U41561), the closest cultivated relative, had a sequence identity of 92-95% only. In contrast, cluster 2 clones were more abundant in the unplanted control (8%) compared to the planted SMFC (2%); these clones fell directly into the radiation of *Geobacter chapellei* (U41561; 95-98% sequence identity). The third cluster, closely related to *Geobacter bremensis* (U96917: 95-99% sequence similarity), included mainly clones from the planted SMFC, however, half as many clone sequences as in cluster 1 (6 %) (Fig. 3a). Myxococcales related sequences were more abundant in the planted SMFC (20%) and the OC control (20%) than in the NP control (10%) and grouped within two larger clusters (cluster 4 and 5) (Fig. 3b). Clones

with an *in silico* T-RF of 129-bp (cluster 4) fell into the larger radiation of *Anaeromyxobacter* spp. with some clones closely related to *Anaeromyxobacter dehalogenans* (AF382400; cluster 4A: ≥95% sequence identity) but the majority of these clones only distantly related (cluster 4B and 4C: 90-95% sequence identity). Cluster 4B clones were more abundant on the anode from planted SMFCs (39% of total cluster 4 sequences) compared with the open circuit control (13%) suggesting that these species might be able to transfer electrons to the anode. Moreover, cluster 4B clones sequences were not detected in the unplanted control indicating a selection of these clone sequences in anode of SMFCs fueled by rice root exudates (Fig. 3b). Cluster 5 clones were only distantly related to *Myxococcus fulvus* (AJ233918; >87% sequence identity) with *in silico* TRFs of 444-bp and 133-bp (Fig. 3b).

A group of clones from the planted SMFC formed a separate cluster within the δ-Proteobacteria (cluster 6) most with T-RFs of 211 bp, 469 bp, 483 bp, 485 bp or 490 bp. Cluster 6 clones were closely related to environmental clone sequences from rice field soil and cluster 6 clones were not detected among controls (NP and OC) (Table 1, Fig. 4b) indicating a strong influence of current and rice root exudates on this population, too. Clone sequences affiliated with the phylum Chloroflexi in current producing SMFCs, both planted (21%) and unplanted (25%), were twice as abundant as in the OC control (12%) and grouped in two clusters (Fig. 3c). The majority of Chloroflexi-related sequences from all samples fell into the Anaerolineae (cluster 7; subphylum I according to Yamada and Sekiguchi (2009)) indicating a stimulation of Anaerolineae populations in current producing anodes. Few clones belonged to the subphylum Dehalococcoidetes (cluster 8; subphylum II according to Yamada and Sekiguchi (2009)) (Fig 3c).

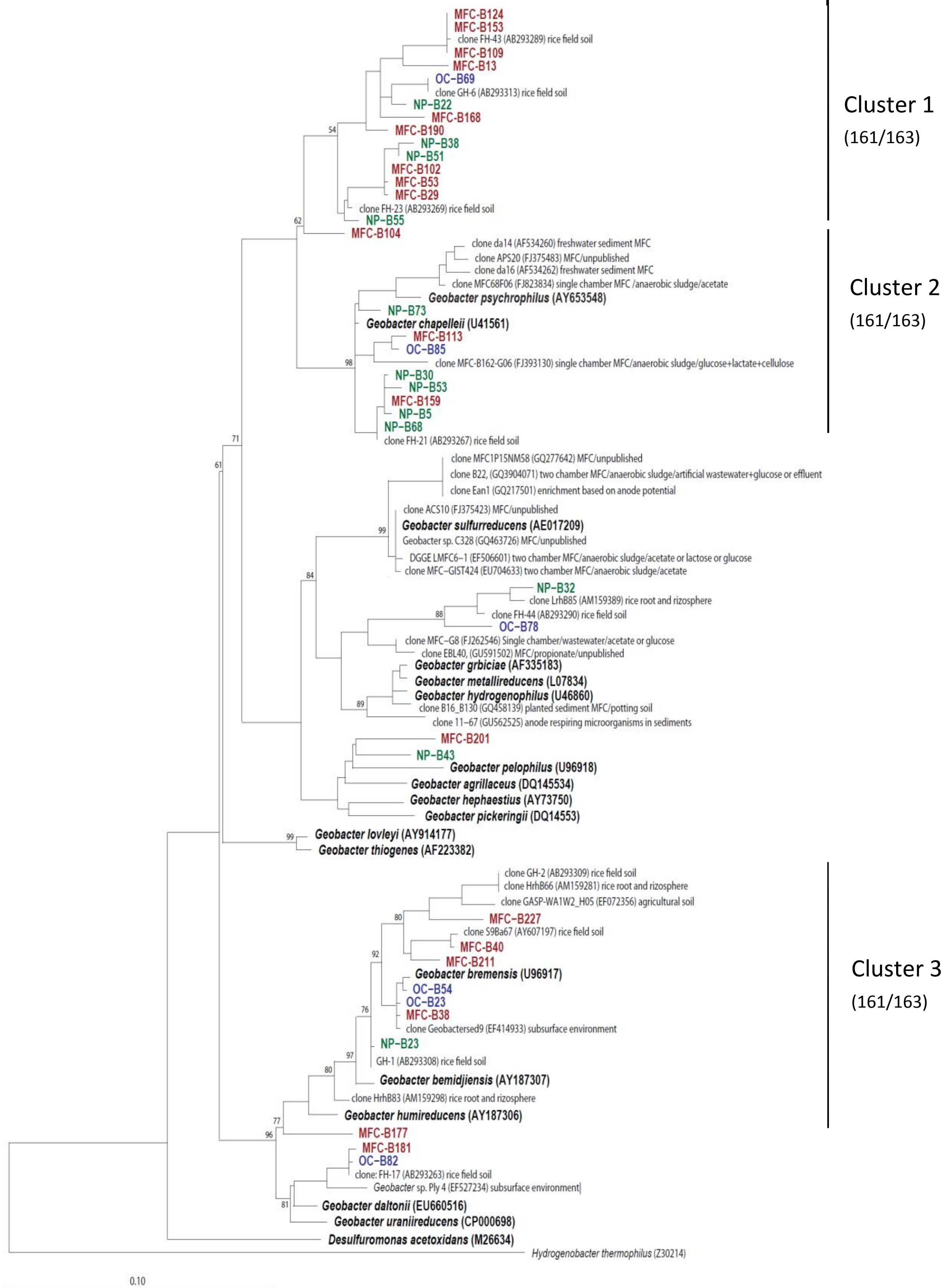
Table 1 Composition of 16S rRNA clone libraries from planted SMFC (MFC-A2), open circuit control (OC-A) and unplanted SMFC (NP-A) and their phylogenetic affiliation.

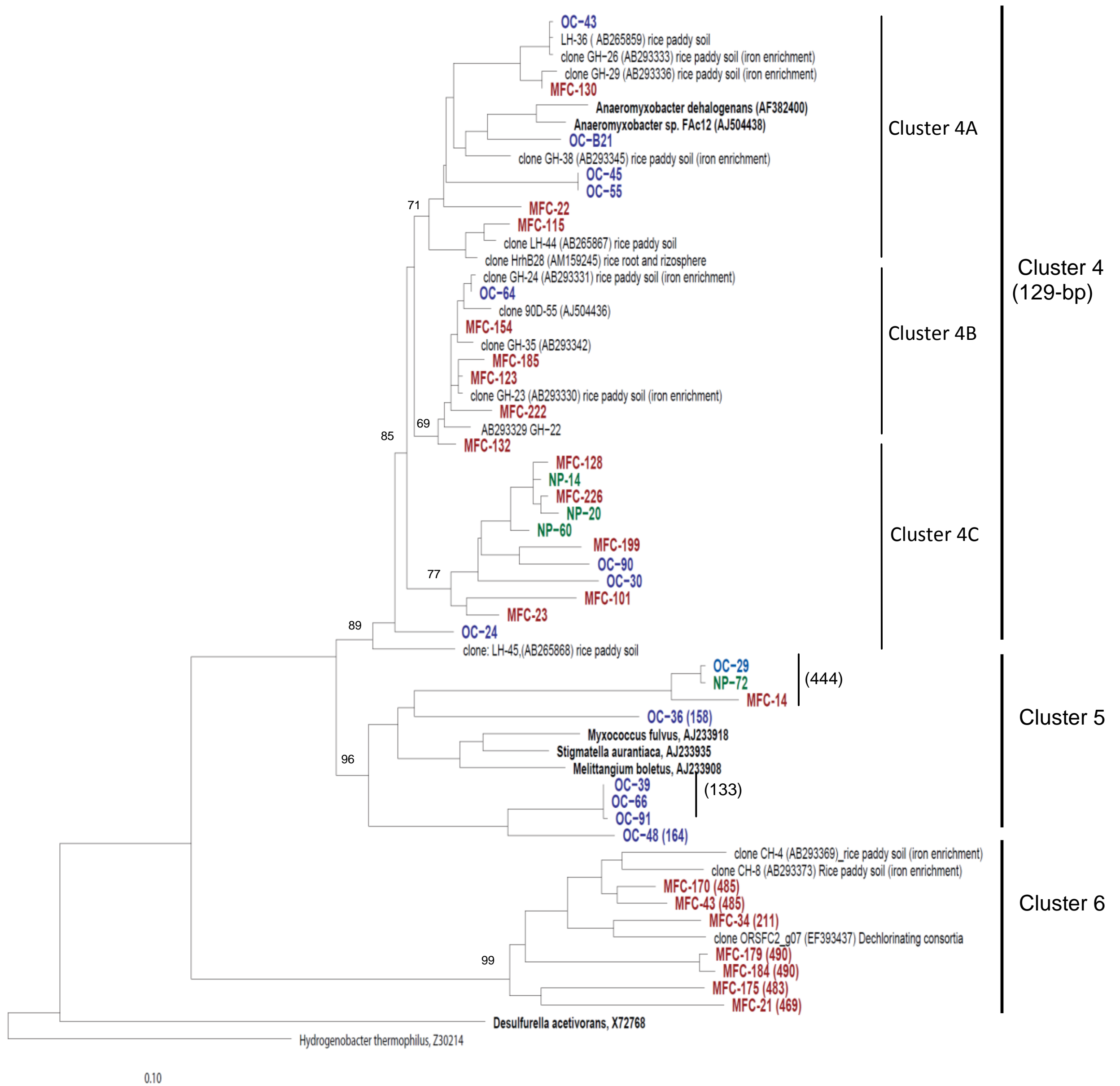
	Clone abundance (%)[a]		
Phylogenetic group[b]	**MFC-A2**	**OC-A**	**NP-A**
δ-Proteobacteria	49.4	32.5	30.6
Geobacteraceae	21.3	7.8	19.3
Unc. *Geobacter* (Cluster 1)	11.2	1.3	6.4
G. chapellei (Cluster 2)	2.2	1.3	8.1
G. bremensis (Cluster 3)	5.6	2.6	1.6
Other Geobacteraceae	2.2	2.6	3.2
Myxococcales	20.2	19.5	9.7
Anaeromyxobacter spp (Cluster 4A)	3.4	5.2	0
Anaeromyxobacter spp (Cluster 4B)	5.6	1.3	0
Anaeromyxobacter spp (Cluster 4C)	5.6	3.9	4.8
Unc. Myxococcales (Cluster 5)	1.1	7.8	1.6
Other Myxococcales	4.5	1.3	3.2
Unc. δ-Proteobacteria (Cluster 6)	7.9	0	0
Other	0	5.2	1.6
Chloroflexi	21.3	11.7	24.2
Anaerolineae (Cluster 7)	16.8	10.4	17.7
Dehalococcoides sp. (Cluster 8)	4.5	1.3	6.5
α-Proteobacteria	6.7	10.4	11.3
β-Proteobacteria	4.5	19.5	11.3
Acidobacteria	3.4	2.6	1.6
Actinobacteria	5.6	7.8	6.4
Bacteroidetes/Chlorobi	1.1	3.9	1.6
Chlamydiae/Verrucomicrobia	1.1	0	3.2
Firmicutes	3.4	10.4	1.6
Gematimonadales	2.2	0	0
γ-Proteobacteria	0	1.3	0
Planctomycetes	0	0	1.6
OP10	1.1	0	6.4

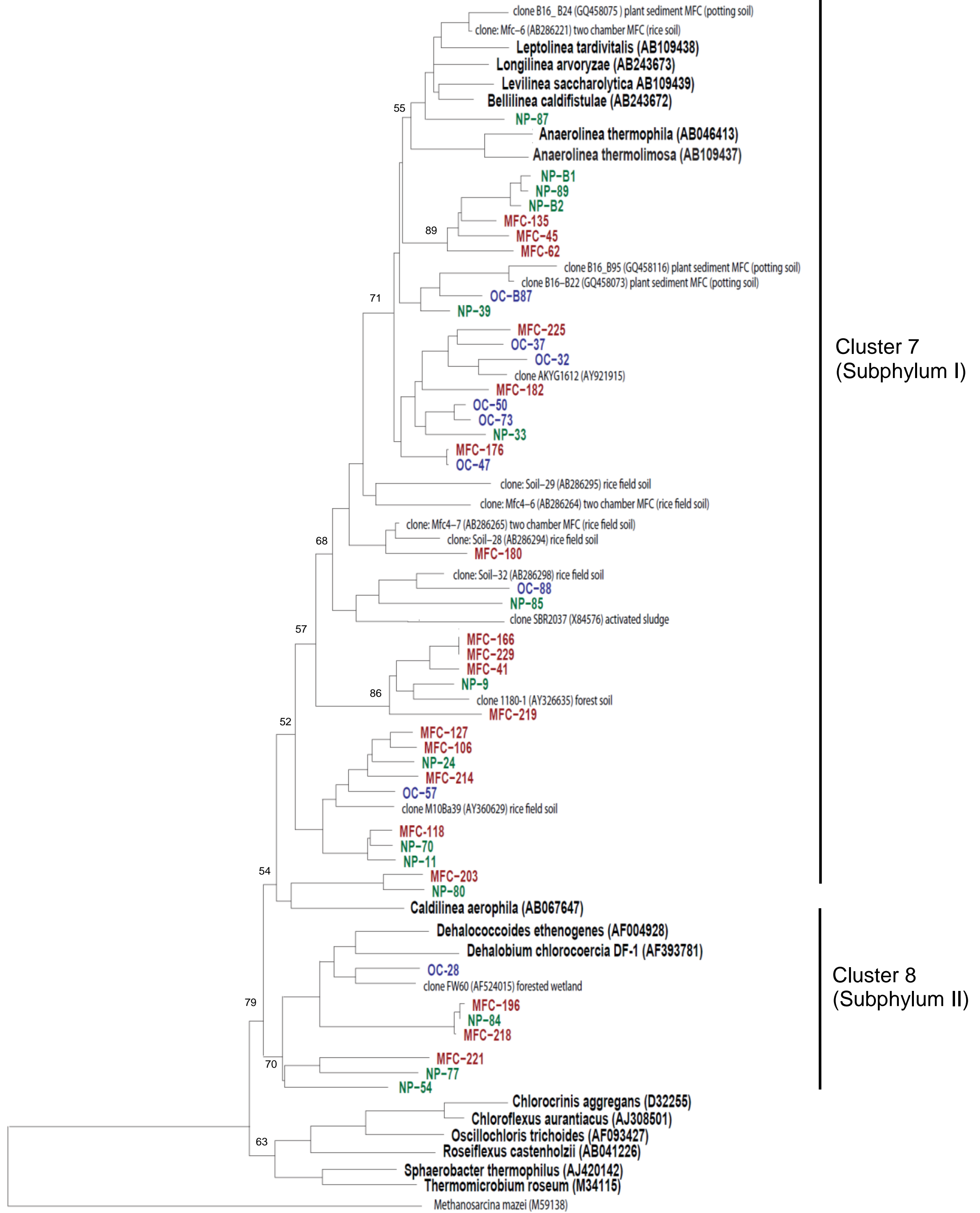
[a]Percentage calculated against total amount of clones (planted MFC n=89; OC-control n=77; NP-control n=62).

[b]The phylogenetic group was assigned according to position in the phylogenetic tree obtained using ARB software

Figure 3 Phylogenetic trees showing the relationships of 16S rRNA clone sequences related to *Geobacter* (a), *Anaeromyxobacter* and uncultured δ-Proteobacteria (b), and Chloroflexi (c). Clones obtained in this study were indicated by MFC (planted SMFC; in red), unplanted control (NP; in green) and open circuit control (OC; in blue). The T-RF sizes are as indicated in brackets in base pairs. Bootstrap values were obtained from 1000 replications. The scale bar represents 10 % sequence divergence. GenBank accession numbers of reference sequences as indicated. Subphylum I and II in the Chloroflexi Phylogenetic tree is according to Yamada and Sekiguchi (2009).


MFC-B124
MFC-B153
clone FH-43 (AB293289) rice field soil
MFC-B109
MFC-B13
OC-B69
clone GH-6 (AB293313) rice field soil
NP-B22
MFC-B168
54
MFC-B190
NP-B38
NP-B51
MFC-B102
MFC-B53
MFC-B29
clone FH-23 (AB293269) rice field soil
NP-B55
62
MFC-B104
Cluster 1
(161/163)
clone da14 (AF534260) freshwater sediment MFC
clone APS20 (FJ375483) MFC/unpublished
clone da16 (AF534262) freshwater sediment MFC
clone MFC68F06 (FJ823834) single chamber MFC /anaerobic sludge/acetate
Geobacter psychrophilus (AY653548)
NP-B73
Geobacter chapellei (U41561)
MFC-B113
OC-B85
98
clone MFC-B162-G06 (FJ393130) single chamber MFC/anaerobic sludge/glucose+lactate+cellulose
NP-B30
NP-B53
MFC-B159
NP-B5
NP-B68
clone FH-21 (AB293267) rice field soil
Cluster 2
(161/163)
71
61
clone MFC1P15NM58 (GQ277642) MFC/unpublished
clone B22, (GQ3904071) two chamber MFC/anaerobic sludge/artificial wastewater+glucose or effluent
clone Ean1 (GQ217501) enrichment based on anode potential
clone ACS10 (FJ375423) MFC/unpublished
99
Geobacter sulfurreducens (AE017209)
Geobacter sp. C328 (GQ463726) MFC/unpublished
DGGE LMFC6–1 (EF506601) two chamber MFC/anaerobic sludge/acetate or lactose or glucose
clone MFC–GIST424 (EU704633) two chamber MFC/anaerobic sludge/acetate
84
NP-B32
clone LrhB85 (AM159389) rice root and rizosphere
88
clone FH-44 (AB293290) rice field soil
OC-B78
clone MFC–G8 (FJ262546) Single chamber/wastewater/acetate or glucose
clone EBL40, (GU591502) MFC/propionate/unpublished
Geobacter grbiciae (AF335183)
Geobacter metallireducens (L07834)
Geobacter hydrogenophilus (U46860)
89
clone B16_B130 (GQ458139) planted sediment MFC/potting soil
clone 11–67 (GU562525) anode respiring microorganisms in sediments
MFC-B201
NP-B43
Geobacter pelophilus (U96918)
Geobacter agrillaceus (DQ145534)
Geobacter hephaestius (AY73750)
Geobacter pickeringii (DQ14553)
99
Geobacter lovleyi (AY914177)
Geobacter thiogenes (AF223382)
clone GH-2 (AB293309) rice field soil
clone HrhB66 (AM159281) rice root and rizosphere
clone GASP-WA1W2_H05 (EF072356) agricultural soil
80
MFC-B227
clone S9Ba67 (AY607197) rice field soil
92
MFC-B40
MFC-B211
Geobacter bremensis (U96917)
OC-B54
OC-B23
76
MFC-B38
clone Geobactersed9 (EF414933) subsurface environment
NP-B23
97
GH-1 (AB293308) rice field soil
80
Geobacter bemidjiensis (AY187307)
clone HrhB83 (AM159298) rice root and rizosphere
77
Geobacter humireducens (AY187306)
Cluster 3
(161/163)
96
MFC-B177
MFC-B181
OC-B82
clone: FH-17 (AB293263) rice field soil
Geobacter sp. Ply 4 (EF527234) subsurface environment
81
Geobacter daltonii (EU660516)
Geobacter uraniireducens (CP000698)
Desulfuromonas acetoxidans (M26634)
Hydrogenobacter thermophilus (Z30214)
0.10


OC-43
LH-36 (AB265859) rice paddy soil
clone GH-26 (AB293333) rice paddy soil (iron enrichment)
clone GH-29 (AB293336) rice paddy soil (iron enrichment)
MFC-130
Anaeromyxobacter dehalogenans (AF382400)
Anaeromyxobacter sp. FAc12 (AJ504438)
OC-B21
clone GH-38 (AB293345) rice paddy soil (iron enrichment)
OC-45
OC-55
MFC-22
MFC-115
clone LH-44 (AB265867) rice paddy soil
clone HrhB28 (AM159245) rice root and rizosphere
clone GH-24 (AB293331) rice paddy soil (iron enrichment)
OC-64
clone 90D-55 (AJ504436)
MFC-154
clone GH-35 (AB293342)
MFC-185
MFC-123
clone GH-23 (AB293330) rice paddy soil (iron enrichment)
MFC-222
AB293329 GH-22
MFC-132
MFC-128
NP-14
MFC-226
NP-20
NP-60
MFC-199
OC-90
OC-30
MFC-101
MFC-23
OC-24
clone: LH-45,(AB265868) rice paddy soil
OC-29
NP-72
MFC-14
(444)
OC-36 (158)
Myxococcus fulvus, AJ233918
Stigmatella aurantiaca, AJ233935
Melittangium boletus, AJ233908
OC-39
OC-66
OC-91
(133)
OC-48 (164)
clone CH-4 (AB293369)_rice paddy soil (iron enrichment)
clone CH-8 (AB293373) Rice paddy soil (iron enrichment)
MFC-170 (485)
MFC-43 (485)
MFC-34 (211)
clone ORSFC2_g07 (EF393437) Dechlorinating consortia
MFC-179 (490)
MFC-184 (490)
MFC-175 (483)
MFC-21 (469)
Desulfurella acetivorans, X72768
Hydrogenobacter thermophilus, Z30214
0.10
71
69
85
77
89
96
99
Cluster 4A
Cluster 4B
Cluster 4C
Cluster 4
(129-bp)
Cluster 5
Cluster 6



clone B16_B24 (GQ458075) plant sediment MFC (potting soil)
clone: Mfc-6 (AB286221) two chamber MFC (rice soil)
Leptolinea tardivitalis (AB109438)
Longilinea arvoryzae (AB243673)
Levilinea saccharolytica AB109439)
Bellilinea caldifistulae (AB243672)
55
NP-87
Anaerolinea thermophila (AB046413)
Anaerolinea thermolimosa (AB109437)
NP-B1
NP-89
NP-B2
MFC-135
89
MFC-45
MFC-62
clone B16_B95 (GQ458116) plant sediment MFC (potting soil)
clone B16-B22 (GQ458073) plant sediment MFC (potting soil)
71
OC-B87
NP-39
MFC-225
OC-37
OC-32
clone AKYG1612 (AY921915)
MFC-182
OC-50
OC-73
NP-33
MFC-176
OC-47
clone: Soil-29 (AB286295) rice field soil
clone: Mfc4-6 (AB286264) two chamber MFC (rice field soil)
clone: Mfc4-7 (AB286265) two chamber MFC (rice field soil)
68
clone: Soil-28 (AB286294) rice field soil
MFC-180
clone: Soil-32 (AB286298) rice field soil
OC-88
NP-85
clone SBR2037 (X84576) activated sludge
57
MFC-166
MFC-229
MFC-41
NP-9
86
clone 1180-1 (AY326635) forest soil
MFC-219
52
MFC-127
MFC-106
NP-24
MFC-214
OC-57
clone M10Ba39 (AY360629) rice field soil
MFC-118
NP-70
NP-11
54
MFC-203
NP-80
Caldilinea aerophila (AB067647)
Dehalococcoides ethenogenes (AF004928)
Dehalobium chlorocoercia DF-1 (AF393781)
OC-28
clone FW60 (AF524015) forested wetland
79
MFC-196
NP-84
MFC-218
MFC-221
70
NP-77
NP-54
Chlorocrinis aggregans (D32255)
Chloroflexus aurantiacus (AJ308501)
63
Oscillochloris trichoides (AF093427)
Roseiflexus castenholzii (AB041226)
Sphaerobacter thermophilus (AJ420142)
Thermomicrobium roseum (M34115)
Methanosarcina mazei (M59138)
0.10
Cluster 7
(Subphylum I)
Cluster 8
(Subphylum II)

Archaeal community diversity and composition

The archaeal diversity on anodes and in soil samples was assessed by T-RFLP and cloning and sequencing of 16S rRNA transcripts. All main archaeal T-RFs were present in all samples analyzed but strong differences were observed in their relative abundances (Fig 1b). Cluster analysis of the T-RFLP profiles showed that SMFC anode samples (planted and unplanted), control anode samples and bulk soil samples grouped in three clusters with an intragroup similarity of approximately 78 % (SMFCs), 85% (open circuit controls) and 95% (bulk soil) (Fig. S2b). PCA showed that this grouping was mainly due to a high abundance of TRFs 86-bp, 440-bp and 293-bp and low abundance of T-RFs 282-bp and 391-bp in the planted SMFC (Fig 1b and Fig S3). T-RFs could be assigned to sequences obtained by cloning and sequence analysis; for example Methanomicrobiales clones had *in silico* TRFs of 82 bp and 391 bp, Methanocellales a T-RF of 391 bp and Methanosaetaceae a T-RF of 282 bp (Table S2).

4.5 Discussion

Bacterial and archaeal diversity

In planted sediment microbial fuel cells (SMFC) the release of root exudates stimulates current production by anode reducing bacteria (De Schamphelaire*, et al.*, 2008). However, the anode reducing bacteria involved in rice field soil SMFCs fueled by rice root exudates had not been identified up to date. Here, microbial communities on anodes of rice field soil SMFC fueled by rice root exudates were compared to those of open circuit and unplanted controls. This comparison should allow discriminating between microorganisms that attach to the anode as a support for growth only from potential current producing bacteria capable of transferring electrons to the anode. We identified *Geobacter* populations, a group of unclassified δ-proteobacteria, Anaerolineae and *Anaeromyxobacter* populations as the predominant bacteria on SMFC anodes by using T-RFLP and cloning/sequencing of 16S rRNA. The analysis of the community compositions by cloning/sequencing of 16S rRNA transcripts (instead of 16S rRNA genes) allows comparing the active populations on the samples analyzed.

A large number of clone sequences related to the larger radiation of *Geobacter* (92-99 % sequence identity to *G. chapellei*) were detected in the planted SMFCs with a clone sequence abundance 3.5 times higher than in open circuit controls (Fig. 1a, Fig. 3a, Table1). Thus *Geobacter* populations appear to be the main current producing bacteria in rice field soil SMFCs fueled by rice root exudates. *Geobacter* spp. have been found abundant on anodes of two chamber MFCs as well as SMFCs (Holmes*, et al.*, 2004, Jung & Regan, 2007) and *Geobacter sulfurreducens* and *G. metallireducens* are known anode reducing bacteria (Bond*, et al.*, 2002, Bond & Lovley, 2003). The release of root exudates into the system selectively enriched a group of uncultured *Geobacter* (cluster 1 clones; 92-95 % sequence identity to *G. chapellei*). These novel electrogenic *Geobacter* populations have not been detected previously on anodes of MFC systems, however, we had identified closely related populations by stable isotope probing with ^{13}C-acetate as iron oxide reducing bacteria from rice field soil previously (Hori*, et al.*, 2010). Our results show that the organic substrates available for oxidation seemed to select for not only *Geobacter* populations in general but for distinct cluster within the Geobacteraceae probably pointing to competition between different *Geobacter* populations indigenously present in rice field soil. In the planted SMFC, cluster 1 *Geobacter* population dominated over other *Geobacter* populations (cluster 2) abundant in the unplanted control. In a two-chamber MFC fed with different substrates (acetate, lactate and glucose) competition between phylogenetically different *Geobacter* populations was observed; even though *G. sulfurreducens* was enriched in most of the anode biofilms it was outcompeted by as yet uncultured *Geobacter* populations (Jung & Regan, 2007).

Cluster 4 clones, falling into the larger radiation of the genus *Anaeromyxobacter* and a group of unclassified δ-Proteobacteria clones (cluster 6), only distantly related to cultured representatives strains within the δ-proteobacteria, were also abundant on SMFC anodes fueled by root exudates. In contrast to *Geobacter* populations, these microorganisms have never been found on anodes of MFCs and therefore, nothing is known about their capacity to transfer electrons to an anode. Clones related to members of the genus *Anaeromyxobacter* were found highly abundant on the anode of planted SMFCs with rice field soil, however, high abundance was also observed in the open circuit control as well as in bulk soil samples. *Anaeromyxobacter* populations have also

been found abundant on rice roots and *Anaeromyxobacter* strain Fac 12, , was isolated as an iron reducing bacterium from rice field soil (Treude*, et al.*, 2003). Recently, we identified *Anaeromyxobacter* populations actively incorporating ^{13}C-acetate by RNA-SIP in the presence of goethite (Hori*, et al.*, 2010), which are closely related (98-99% sequence identity) to those *Anaeromyxobacter* clones (cluster 4A and 4B; Fig. 3b) found on the anode. As the capability of reducing iron does not necessary imply the ability of electron transfer to anodes (Richter*, et al.*, 2007), *Anaeromyxobacter* isolates have to be tested for an unequivocal proof of anode-reducing capability. In current producing SMFCs, the presence of root exudates enriched for a group of *Anaeromyxobacter* populations with sequence identity of 92-95% to *A. dehalogenans* (cluster 4B, fig 3b). Cluster 4B related bacteria were stimulated by root exudates and might be coupling current production to rice root exudate oxidation.

The novel δ-Proteobacteria clones (cluster 6) detected abundantly on the anode of planted SMFCs (17% of all clones) were the only group of clones not detected in any of the controls strongly indicating an involvement of these microorganisms in current production coupled to rice root exudation.

Besides δ-Proteobacteria, which are known to play a role in current production from MFCs, we detected Anaerolineae on anodes of SMFCs. Twice as many Anaerolineae sequences were found on the anode of the planted and unplanted SMFC compared to open circuit controls. This suggests that Anaerolineae might be important in current production, however, they were apparently not stimulated by root exudates. Anaerolineae have scarcely been found in MFCs; they appear to be important in MFC inoculated with soil, in particular rice field soil. With potting soil as substratum in SMFCs we detected 6 % of all clones were Anaerolineae (De Schamphelaire*, et al.*, 2010) however, their proportion in rice field soil (17% of all clones) was much higher. Furthermore, Ishii *et al.* (2008) observed an enrichment of up to 22% of Chloroflexi sequences on the anode of a two chamber MFC inoculated with rice field soil. The Anaerolineae lineage still contains surprisingly diverse, yet to be cultured, environmental clade (Yamada & Sekiguchi, 2009). The cultured representative of these lineage are filamentous, slow growing, aerobic and anaerobic heterotrophs decomposing carbohydrates and aminoacids (Yamada & Sekiguchi, 2009). Possibly, the uncultured Anaerolinea found here might be involved in electron transfer to anodes.

Members of the β-Proteobacteria, α-Proteobacteria and Firmicutes, abundant in open circuit controls, decreased in the current producing MFC and even though these groups have been suggested to be important in other MFC systems (Kim*, et al.*, 2007, Rismani-Yazdi*, et al.*, 2007, Ishii*, et al.*, 2008, Ishii*, et al.*, 2008, Chae*, et al.*, 2009, Chung & Okabe, 2009, Patil*, et al.*, 2009), including planted SMFC (Kaku*, et al.*, 2008), they were not key players in direct current production in our system.

The methanogenic Archaea in rice field soil produce approximately 13 % of the global CH_4 emissions and therefore, rice paddies are a major source of atmospheric methane, a known green house gas (Lelieveld*, et al.*, 1998). The effects of rice field soil SMFC on the archaeal community composition might also affect the emission of methane; however, no studies have been performed in this direction. Here, we observed a change of the archaeal community composition on the anodes of SMFCs compared to open circuit controls and bulk soil samples. The decrease of *Methanosaeta* spp. (Fig. 1b, Fig. S3), a strictly acetoclastic methanogens, on the anode of rice field soil SMFCs could be due to the competition with anode reducing *Geobacter* populations for the common substrate acetate. The concomitant increase of Methanobacteriales (Fig. 1b, Fig S3) could indicate a change from acetotrophic to hydrogenotrophic methanogenesis. Ishii *et al*. (2008) detected less methanogens and suppressed methanogenesis on the anode of a two-chambered MFC inoculated with 1 % rice field soil compared to open circuit control anodes. However, not much is known about current production by Archaea. Here, we found an increase of the relative abundance of two unknown archaeal T-RFs (293-bp and 440-bp) in the planted SMFC anode sample compared to the OC control anode and it has been previously shown that some methanogens are able to reduce iron and humic acids (Bond & Lovley, 2002). Therefore, Archaea might also be involved in current production in MFCs. Moreover, a recent study showed that methanogens are able to accept electrons from a cathode to produce methane indicating an ability of methanogens to directly interact with electrodes (Cheng *et al.*, 2009).

Ecology of rice planted SMFC anodes

Current generation in our planted SMFCs was apparently mainly driven by δ-Proteobacteria and Chloroflexi. In a previous study we used potting soil as support for

rice plants in SMFCs (De Schamphelaire*, et al.*, 2010) and *Desulfobulbus*-related spp. were the most abundant bacteria found on anodes. *Desulfobulbus propionicus* is able to transfer electrons directly to the anode, however, electrons originate from the oxidation of S° (to SO_4^{2-}), propionate, lactate and pyruvate; but *D. propionicus* cannot oxidize acetate, which is likely to be the primary electron donor for electricity production in SMFCs (Holmes*, et al.*, 2004). Here, we used rice field soil as inoculum for the planted SMFCs and *Geobacter* populations were most abundant, probably oxidizing acetate transferring electrons directly to the anode (Schroder, 2007). The type of anode reducing bacteria responsible for current production might affect the efficiency of the SMFCs as electron transfer to the electrode from oxidation of pyruvate, lactate, and propionate by *D. propionicus* is inefficient and only ca. 25% of the electrons available from the incomplete oxidation were transferred to the electrode surface (Holmes*, et al.*, 2004). On the contrary, *Geobacter* spp. have been shown to produce high current densities with high coulombic efficiencies (Nevin*, et al.*, 2008, Yi*, et al.*, 2009). The indigenous microbial community present in the SMFC inoculum is probably a critical factor determining the microbial community that will develop on anodes which could affect the efficiency of the planted SMFC. In rice planted SMFCs with potting soil as substratum the anode was dominated by *Desulfobulbus* populations while when rice field soil was used as substratum, *Geobacter* populations were predominant.

In planted SMFCs, current is enhanced by the release of root exudates into the soil. However, which compounds are used directly by anode reducing bacteria is not known. Our results showed that some sequence clusters of *Geobacter* and *Anaeromyxobacter* were stimulated when root exudates were produced. However, other highly abundant bacteria such as members of the Anaerolineae were stable and independent from root exudate production. One possibility is that current is produced from both root exudates and intermediate degradation compounds such as acetate and therefore, different populations were selectively enriched on the anode reflecting substrate diversity. The diversity of electron donors used within the genus *Geobacter* is high; for example, *G. bremensis* is able to use a larger variety of organic compounds as electron donor than *G. chapellei* (Coates*, et al.*, 2001, Straub & Buchholz-Cleven, 2001, Lovley*, et al.*, 2004).

Rice root exudates comprise a complex pool of compounds including carbohydrates, amino acids, fatty acids and others (Grayston*, et al.*, 1997). Acetate is a key intermediate in the degradation of organic matter in rice field soils (Yao & Conrad, 1999). *Geobacter* spp. are capable of current production from acetate oxidation (Bond & Lovley, 2003, Schroder, 2007) and *Anaeromyxobacter* spp. are able to oxidize acetate with concomitant reduction of iron (Hori*, et al.*, 2010). The cultured representatives of Anaerolineae are aerobic and anaerobic heterotrophs decomposing carbohydrates and amino acids (Yamada & Sekiguchi, 2009). An anaerobic "food chain" involving degradation of complex organic matter (root exudates) into acetate followed by acetate oxidation with concomitant current production is probably occurring on anodes of rice planted SMFCs. One possibility could be that Anaerolineae populations degrade carbohydrates and amino acids released through the root producing acetate which is used by *Geobacter* and *Anaeromyxobacter* populations to produce current. However, we cannot dismiss the possibility that some populations (for example, cluster 1 *Geobacter* and cluster 4B *Anaeromyxobacter*) might also be able to directly produce current from root exudates (for example, from organic acids released by the rice plant).

4.6 Conclusions

Differential analysis of planted SMFCs, unplanted and open circuit controls, allowed to delineate populations selectively enriched on anodes of SMFCs fueled by root exudates. A predominance of *Geobacter* populations but also a group of unclassified δ-Proteobacteria as well as *Anaeromyxobacter* populations and Anaerolineae were detected on anodes of rice field soil SMFCs fueled by root exudates. The release of root exudates into the system selectively enriched for distinct populations of *Geobacter*, *Anaeromyxobacter* and unclassified δ-Proteobacteria. However, it is still not clear whether root exudates might be directly converted into current or first degraded into intermediate compounds like acetate by fermenting bacteria which would then serve as fuel for current production. Moreover, competition for the intermediate compound acetate seems to occur as a decrease of Methanosarcinales in current producing SMFCs was observed. When current was produced clear changes in the bacterial and archaeal community compositions were observed and factors such as plant presence and

inoculum seem important factors determining the active bacteria on anodes. Further experiments, e.g. using a stable isotope probing approach, would be required to get more insight into the interaction between root exudates and anode reducing bacteria.

4.7 References

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4.8 Supplementary data

Figures

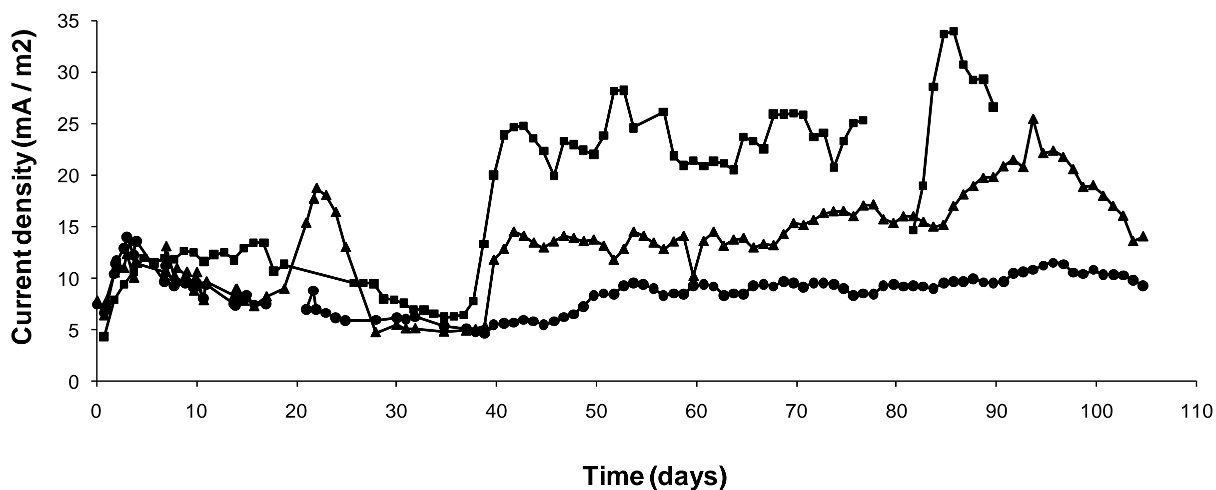

Figure S1 Current density (mA/m^2TAS) profiles of planted (filled square: MFC-A, filled triangles: MFC-B) and unplanted control (filled circle: NP-A) during operation time.

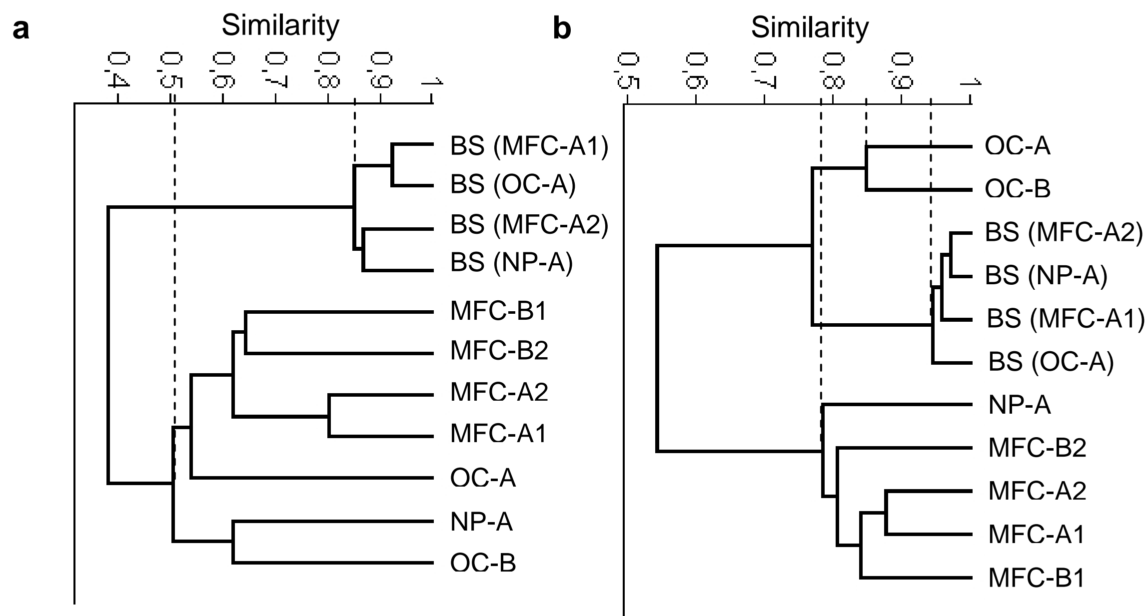

Figure S2 Cluster analysis for a) bacterial and b) archaeal T-RFLP profiles for anode samples and bulk soil samples from planted MFCs (MFC-A and MFC-B), open circuit controls (OC-A and OC-B), unplanted control (NPA) and bulk soil samples (indicated with BS). Cluster analysis was performed with PAST software using the algorithm UPGMA and the Bray-Curtis similarity index. Dotted lines indicate the intragroup similarities.

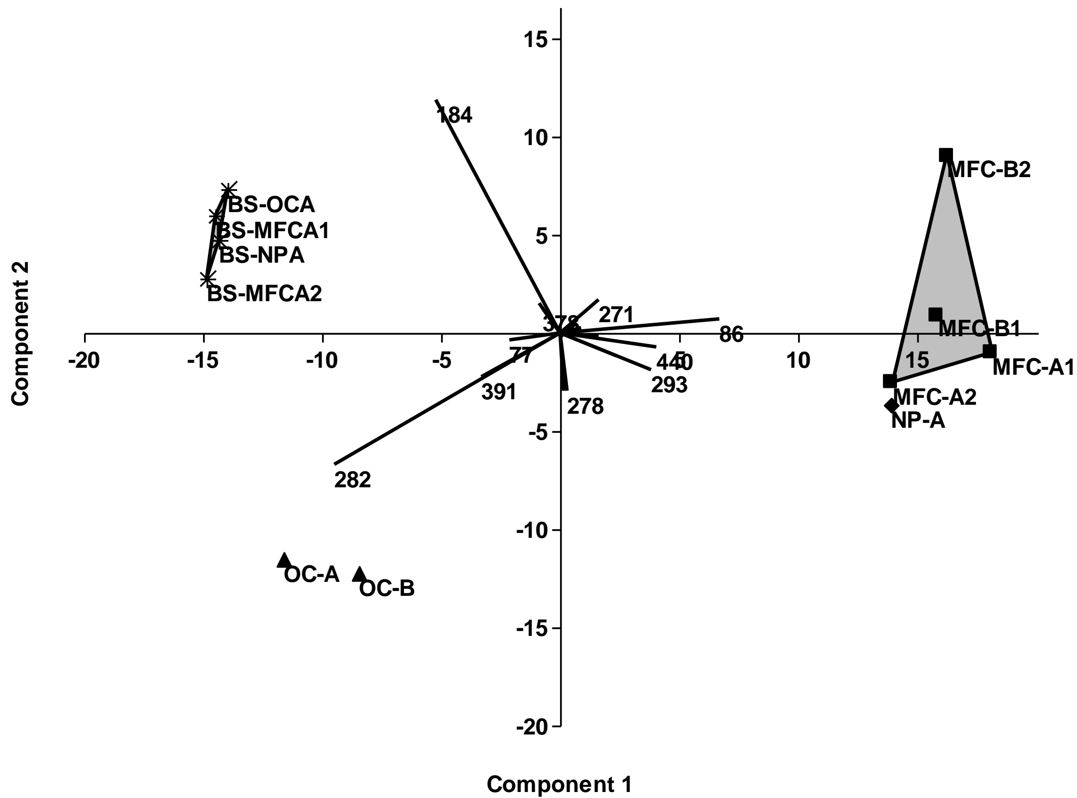

Figure S3. Principal component analysis (PCA) of archaeal T-RFLP profiles from plant SMFC (closed squares), open circuit controls (closed triangles), unplanted control (closed diamond) and bulk soil samples (star). The clustering reflects differences in the T-RFLP profiles. The vectors shown indicate the T-RFs which explain the clustering of the samples. The smallest convex polygon containing all points is shown. Components 1 and 2 explain 88.47 % of the variance.

Tables

Table S1. Main bacterial T-RFs relative abundance (T-RF), their phylogenetic assignment and amount of 16S rRNA clones (N) determined by *in silico* restriction in planted SMFC (MFC-A2), unplanted control (NP), open circuit control (OC) and bulk soil samples (BS).

	MFC-A2 (%[a])		OC (%)		NP (%)		BS (%)	
T-RFs	**T-RF**	**N[b]**	**T-RF**	**N**	**T-RF**	**N**	**T-RF**	**Phylogenetic assignment[c]**
59	1	1	2	1	2	0	6	Chloroflexi
72	7	ND[d]	6	ND	5	ND	4	Unidentified
88	2	0	2	1	2	2	3	Bacteroidetes/Chlorobi
121	2	4	1	3	3	2	0	α-Proteobacteria
124	20	13	14	6	12	5	11	*Anaeromyxobacter* sp.
136	3	1	1	0	4	2	3	Actinobacteria & α-Proteobacteria
138	1	1	3	4	4	2	4	unc. *Myxococcales* & OP10
147	3	1	4	5	7	2	7	Actinobacteria
148	4	2	6	4	6	6	8	α-Proteobacteria
153	3	ND	1	ND	1	ND	0	Unidentified
154	3	1	3	3	2	0	1	*Bacillales*
158	1	2	0	5	0	0	6	*Anaeromyxobacter* sp.
159/161	24	20	9	8	8	18	0	*Geobacter* spp.
199	2	1	3	1	3	2	5	Acidobacteria & Chloroflexi
207	3	ND	2	ND	2	ND	0	Unidentified
268	0	1	1	3	0	2	7	Firmicutes & Actinobacteria
436	0	1	4	1	4	2	0	unc. δ-Proteobacteria
487	0	0	2	3	2	2	1	β-Proteobacteria
489	1	3	3	4	0	0	0	β- & unc. δ-Proteobacteria
511	1	2	2	3	1	2	9	Chloroflexi
516	0	1	0	0	0	5	2	Chloroflexi
520	0	3	0	4	0	0	3	Clostridium & Chloroflexi
526	1	0	2	1	3	2	4	Chloroflexi
Other[e]	18	18	26	20	28	24	17	

[a]Percentages were calculated against total T-RF relative abundances or total 16S rRNA clones.
[b] % of 16S rRNA clones with *in silico* T-RFs assigned to real T-RFs.
[c]Phylogenetic affiliation was determined by using ARB software.
[d]ND = not detected; corresponds to T-RF which could not be assigned by in silico restriction of 16S rRNA clone sequences.
[e]T-RFs with less than 1 % relative abundance and *in silico* T-RFs present in only one clone sequence were grouped in "Other".

Table S2. Phylogenetic affiliation of archaeal 16S rRNA clone sequences from the planted SMFC anode sample (MFC-A2) and T-RFs assignment based on *in silico* analysis of 16S rRNA sequences.

Phylogenetic group	Clones (%) (n = 62)	T-RF (bp)
Methanosarcinales	34	184
Methanosaetaceae	26	282
Methanocellales (Rice cluster I)	16	391
Methanomicrobiales	15	82 (8)[a], 391(7)
Rice cluster IV	4	738 (2), 201 (2)
Unc. Crenarchaeote	4	390 (2), 184(2)
Unc. Euryarchaeote	2	390
Unc. Thermoplasmatales	2	379

[a]The number of clones with a certain T-RF length is indicated in brackets when more than two T-RFs were detected within a phylogenetic group.

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Chapter 5

Identification of β-Proteobacteria and Anaerolineae as Active Populations Degrading Root Exudates on Sediment Microbial Fuel Cell Anodes

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Author contributions

AC and MWF designed the study. AC constructed and operated sediment microbial fuel cell. AC performed stable isotope probing, sampling, molecular analysis and GC measurements. AC performed all data analysis. AC wrote the manuscript.

5.1 Abstract

Root exudates are released into rice field soils and are actively degraded by rhizospheric microorganisms. In planted sediment microbial fuel cells (SMFC) root exudates are used as fuel for current production. Here, the active bacterial community on anodes, bulk soil and roots of SMFCs fueled by rice root exudates was assessed by 454-pyrosequencing of 16S rRNA. Furthermore, stable isotope probing (SIP) using ^{13}C-CO_2 combined with terminal restriction fragment length polymorphism (T-RFLP) and 454-pyrosequencing of 16S rRNA, allowed determining which bacteria actively used rice root exudates on anodes of planted SMFC and OC controls. *Geobacter* spp. (13%) and *Anaeromyxobacter* spp. (5%) within the δ-Proteobacteria and Anaerolineae (6%) were the most active bacteria on anodes from SMFC fueled by root exudates. *Anaeromyxobacter* spp. were also active on open circuit controls (6%) and SMFC roots (14%). rRNA SIP showed that the active populations degrading root exudates on the anode of planted SMFCs belonged to β–Proteobacteria and Chloroflexi. *Acidovorax* spp., Oxalobacteraceae, *Dechloromonas* spp. and Anaerolineae were the actively incorporating carbon from rice root exudates on planted SMFC anodes. This indicated that the main current producing populations, for example, *Geobacter* spp., did not assimilate ^{13}C labeled root exudates.

5.2 Introduction

Plants continuously provide an input of organic matter to the soil via their roots. This process is called rhizodeposition and rhizodeposits comprise water-soluble exudates, secretions, lysates, mucilages, sloughed-off cells and decaying roots (Lu, 2003). Since it is difficult to experimentally distinguish root exudates from other rhizodeposits, they are often defined as all organic substances released into the environment by healthy and intact roots. They comprise carbohydrates, amino acids, amides, aliphatic acids, aromatic acids, fatty acids, sterols, enzymes, hormones, vitamins, and others (Grayston, *et al.*, 1997). Organic substances released from rice roots serve as an important carbon and energy source for microbial activities in the rhizosphere. The microbial dynamics are therefore significantly affected by photosynthate inputs. In wetland soils, plant-derived

organic substances additionally serve as an important carbon source for CH_4 production and emission (Dannenberg & Conrad, 1999, Kimura*, et al.*, 2004).

Microbial fuel cells (MFCs) are devices, where chemical energy is directly converted to electrical energy by anode reducing microorganisms (Logan*, et al.*, 2006, Lovley, 2006, Davis & Higson, 2007). In sediment microbial fuel cells (SMFCs), the anode is buried into a sediment or soil and the cathode is positioned in the overlying water layer (Tender*, et al.*, 2002). Current is generated due to the activity of anode reducing bacteria, which oxidize organic matter and use the anode as final electron acceptor. The electrons travel to the cathode through an electrical circuit where they reduce oxygen to water, and so, current is produced (Rabaey & Verstraete, 2005). It was recently demonstrated that electric energy can be harvested from planted SMFCs where rhizodeposits are used as fuel (De Schamphelaire*, et al.*, 2008, Kaku*, et al.*, 2008, Strik*, et al.*, 2008). Planted SMFCs are an adaptation of SMFCs, where the anode is buried into a sediment or soil closely in contact to the rhizosphere. We have shown that plants influence not only the amount of current produced but also the microbial populations on anodes (De Schamphelaire*, et al.*, 2010). Moreover, we found that *Desulfobulbus* related spp. (De Schamphelaire*, et al.*, 2010), *Geobacter* spp., *Anaeromyxobacter* spp. and *Chloroflexi* (chapter 3) were abundant on anodes of planted SMFCs.

One of the major challanges of microbial ecology is linking the microbial community with its function. A direct way of linking identity of microorganisms to a specific function is stable isotope probing (SIP) of nucleic acids (Radajewski*, et al.*, 2000), and in particular of RNA (Manefield*, et al.*, 2002). Nucleic acid SIP capitalizes on the incorporation of heavy stable isotopes (^{13}C, ^{15}N, ^{18}O) into RNA (or DNA), the physical separation of labeled, isotopically ‘heavy’ RNA from unlabeled, ‘light’ RNA, and subsequent identification of actively label incorporating populations. For example, this technique has been used previously to identify the active Bacteria and Archaea on rice roots by administrating ^{13}C-CO_2 to rice plants (Lu & Conrad, 2005, Lu*, et al.*, 2006). The authors were able to identify α-Proteobacteria and β-Proteobacteria as the responsible microorganisms for root exudate degradation and Rice cluster I Archaea as the active methanogens on rice roots. The identification of labeled “heavy” rRNA from SIP centrifugation gradients is generally performed by combining profiling techniques (DGGE and T-RFLP) with cloning of 16S rRNA gene amplicons and their sequencing by the

Sanger method (Lueders*, et al.*, 2004, Lu*, et al.*, 2006). One of the potential limitations of SIP which might affect identification of the target microorganisms is the dilution of the labeled substrate before its assimilation and incorporation (Manefield*, et al.*, 2002). For RNA-SIP and DNA-SIP, the synthesis of labeled nucleic acid must be sufficient for the separation and detection of labeled molecules from the abundant background of unlabeled material (Neufeld*, et al.*, 2007).

Anodes from rice planted SMFCs are an interesting ecosystem and in our previous work (chapter 3) we detected the presence of several bacterial groups on the anode and identified the main current producing bacteria. However, no comparison with microbial communities on roots or bulk soil was performed which could help to understand the microbial processes in the different compartments of the system. Moreover, rice root exudates stimulated certain bacterial groups however, no confirmation on the use of root exudates directly for current was obtained. In the present work the bacterial community composition on anodes, bulk soil and roots of SMFCs fueled by root exudates were studied by 454-pyrosequencing. Furthermore, by combining ^{13}C-CO_2 rRNA SIP with 454-pyrosequencing and T-RFLP, the anode bacteria actively degrading root exudates on anodes were identified.

5.3 Materials and Methods

Microbial fuel cell operation, ^{13}C-CO_2 incubation and geochemical analysis

Planted sediment microbial fuel cells (SMFC) were operated from July-September 2009 (51 days) and included five replicate SMFCs and triplicate open circuit (OC) controls. The MFCs were constructed using plastic containers which were filled with 3 kg of rice field soil. The soil was sampled in 2009 from a drained rice field of the Italian Rice Research Institute “Instituto Sperimentale per la Cerealicoltura” near Vercelli (Po River valley, Italy). Soil parameters were described previously (Chin and Conrad, 1995). The soil was air dried and stored at room temperature and preparation of the soil and sieving (mesh size, 5 mm) were done as previously described (Chin and Conrad, 1995). The soil was flooded with water leaving a layer of overlying water of 5 cm. In each planted SMFC and

OC controls two anodes of 25 cm by 8 cm and 12.5 cm by 8 cm were placed vertically forming a cylinder in the soil matrix (Total anode surface (TAS): 634 cm^2) and a cathode with a total surface of 254 cm^2 was placed in the overlying water layer. The anodes and cathodes were made of carbon felt (Alfa Aesar, Ward Hill, USA; 3,18mm thick), interwoven with graphite rods (5mm diameter, Thielmann Graphite GmbH, Grolsheim, Germany) and attached to the electrical circuitry through insulated connections. The electrical circuit was closed (except for the open circuit controls) through an external resistance of 100 Ω. Three one week old plants (*Orzya sativa* cultivar Koral) were planted in each pot and fertilizer (urea (45g/L), Na_2HPO_4 x $2H_2O$ (17g/L) and KCl (50g/L); 10 mL / 2Kg soil) was added twice at the beginning of the operation. MFCs were operated in a greenhouse facility with light:dark cycles of 12h:12h at an average temperature of 25 °C. The potential (mV) was recorded every 15 minutes with a datalogger (Agilent 34970A, Agilent Technologies, Böblingen) and current and power densities were calculated as reported previously (Logan*, et al.*, 2006). From day 44 to 51 of operation planted SMFCs were pulse-labeled during the light period. For this, plants from three SMFCs and two OC controls were covered with a transparent Plexiglas cylinder and 35mL of ^{13}C-CO_2 were added every one hour, 8 times per day during 8 days (57 times in total). Two planted SMFC and one OC control served as unlabeled controls and ^{12}C-CO_2 was added in exact same conditions as the labeled setups. To produce 1L of CO_2 8 mL of H_3PO_4 50% were added to 3.4 g of 13C or 12C $NaHCO_3$ (99.9 %) and the produced $CO_2$was stored in gas tight bags (SKC Inc, USA). During the night, chambers were removed in order to avoid accumulation of unlabeled CO_2. Total CH_4 and CO_2 in chamber and pore water samples were analyzed by gas chromatography (Roy*, et al.*, 1997) and the ^{13}C atoms percent of CH_4 and CO_2 was determined by GC-isotope ratio mass spectrometry (Conrad*, et al.*, 2000). Volatile fatty acids from duplicate pore water samples taken once per day during the pulse-labeling were measured by high-pressure liquid chromatography (Krumbock & Conrad, 1991). After the pulse-labeling period, anodes, roots and bulk soil were sampled and stored at -80°C for further molecular analysis.

Molecular analysis of bacterial communities

RNA extraction and isopycnic centrifugation

4 parallel RNA extractions with 0.5g of anode material, bulk soil or root material were performed using a bead beating protocol as described previously by Lueders *et al*. (2004). RNA was quantified using the Ribogreen RNA-quantification kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. Extracted RNA (500ng) was density separated by isopycnic centrifugation in cesium trifluoroacetate (Amersham Biosciences, Freiburg, Germany). Gradients of density-resolved RNA were fractionated, the cesium trifluoroacetate buoyant density (BD) of each fraction determined, and RNA precipitated from fractions as described earlier (Lueders*, et al.*, 2003).

Terminal fragment restriction length polymorphism (T-RFLP)

RNA from each density fraction of each treatment was used as a template for reverse transcription-PCR (RT-PCR) using a single step RT-PCR system (Access Quick, Promega, Mannheim, Germany) for terminal restriction fragment length polymorphism (T-RFLP) profiling. 5' 6-carboxyfluorescein labeled (FAM) primers were used to specifically amplify *Bacteria* (FAM-Ba27f and Ba907r). PCR products were cleaned up (GenElute™PCR Clean-Up Kit, Sigma-Aldrich) and ~100 ng were digested with restriction enzymes *Msp*I (Promega). Purified digests (1-2 µl) (SigmaSpin™ Post-Reaction Clean-Up Columns, Sigma-Aldrich) were mixed with 11 µl of formamide (Hidi; Applera Deutschland GmbH, Darmstadt) and 0,3 µl molecular weight marker (X-Rhodamine MapMarker® 1000, BioVentures, Murfreesboro, Tennessee, USA), and denaturized for 3 minutes at 95°C. Electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer (Applied biosystems). T-RFLP electropherograms were analyzed with GeneMapper Software 4.0 (Applied Biosystems). Tables were extracted for each sample with peak size vs. fluorescence intensity and TRFs that differed by ±1 bp in different profiles were considered as identical in order to compare the T-RFLP profiles between different samples. The peak heights were standardized to the minimum sample according to Dunbar *et al* (2000). The relative abundance of each T-RF within a given T-RFLP pattern

was calculated as the peak height of the respective T-RF divided by the total peak height of all T-RFs detected within a fragment length range between 50 and 900 bp. Changes in T-RF relative abundances in the different fractions were analyzed constructing graphs with T-RF relative abundance vs. buoyant density of the fractions.

Next generation sequencing

The 16S rRNA from “heavy” and “light” gradient fractions, collected after isopycnic separation, of labeled and unlabeled SMFCs and open circuit controls were used for 454-pyrosequencing as well as roots and bulk soil from a labeled planted SMFC. Complete cDNA was synthesized using ImProm-II™ Reverse Transcriptase (Promega) by the following procedure: 10 ng of purified RNA and 0.5 mM of random hexamer primers were incubated at 70°C for 5 min followed by 5 min chilling on ice. The following mixture was then directly added to the tubes: 4 ml of 5x ImProm-II™ Reaction Buffer, 3 mM MgCl2, 0.5 mM of each dNTP, 20 ng of bovine serum albumin (BSA) (Roche) and 20 U of Recombinant RNasin Ribonuclease Inhibitor (Promega). The reaction tubes were incubated at 25°C for 5 min followed by 52°C for 1 h, and then denaturation at 70°C for 15 min. The resulting cDNA product was used directly as a template for the PCR reactions needed for pyrosequencing. Fragments of 16S rRNA genes were amplified from the cDNA, with primer sets, 27F (5’-GAGTTTGATCMTGGCTCAG) and 518R (5’-GTTACCGCGGCTGCTGG) with 10 different barcodes to sort each sample from the mixed pyrosequencing outcomes. PCR reactions were conducted in quadruplicate of 50 µl each which were later combined to minimize reaction bias. Each PCR reaction contained 10µL of 5x Herculase II Reaction Buffer (Eurofins MWG Operon), 12.5 µM of each primer (Agilent Technologies), 1 µl of Herculase II Fusion DNA polymerase (Agilent Technologies) and 1µl of template. The following program was used: 95°C for 2 min followed by 25 cycles of 94°C for 30 sec, 55°C for 30sec and 68°C for 1 min and a single step of final elongation at 68°C for 5 min. After amplification, the quadruplicate PCR reactions were pooled and loaded on 1% agarose gel stained with ethidium bromide. PCR products were cleaned up (GenElute™PCR Clean-Up Kit, Sigma-Aldrich) and quantified by Micro-Volume UV-Vis Spectrophotometer NanoDrop (Thermo Scientific). Amplicon pyrosequencing was performed by GATC (Germany) using a 454/Roche GS-FLX Titanium instrument (Roche, NJ, USA). Equal amounts of ten samples were pooled and their sequences separated according to barcodes.

Sequence analysis, phylogenetic classification and T-RF assignment

Raw sequencing reads were quality trimmed according to published recommendations (Huse*, et al.*, 2007) using the RDP Pyrosequencing Pipeline (Cole*, et al.*, 2009) applying the following criteria: i) exact matches to primer sequences; ii) no ambiguous bases; iii) read-lengths not shorter than the 150 bp. For large scale assignments into the new Bergey bacterial taxonomy (Garrity*, et al.*, 2004) we used the Naïve Bayesian Classifier (RDP-classifier; http://pyro.cme.msu.edu/), which provides rapid taxonomic classifications from domain to genus of both partial and full-length rRNA gene sequences along with bootstrap like confidence estimates (Wang*, et al.*, 2007). The results from the RDP classification were imported into excel and relative sequence abundance at Phylum and genus levels were compared between “heavy” and “light” fractions of the different samples and between the different SMFC compartments. Pyrosequencing reads were aligned using Infernal (Nawrocki & Eddy, 2007) and associated covariance models obtained from the Ribosomal Database Project Group. By applying the furthest neighbour approach using the Complete Linkage Clustering application of the RDP pyrosequencing pipeline, trimmed pyrosequencing sequences could be assigned to phylotype clusters of 95% identity. Based on these clusters, rarefaction curves (Colwell & Coddington, 1994), Shannon diversities (Gotelli, 2002) and Chao1 richness estimations (Chao & Bunge, 2002) were calculated using RDP pipeline. For T-RF assignment, the predominant *in silico* restriction fragment sizes (*in silico* T-RFs) were obtained by searching the restriction site of the restriction enzyme *Msp*I (C|CGG) in representative sequences (>450-bp) from the 60 most abundant clusters, from each sample.

5.4 Results

Electrochemical performance and CO_2 turnover in planted SMFCs

The planted SMFCs with rice field soil were operated for nearly two months. During this period the current output varied presenting a steep increase during the first 5 days followed by a slow decrease until day 20, where a steep increase occurred followed by a constant current production period after day 25 (Fig. 1). During the stable current output

periods current densities were similar in all SMFC (20.0 ± 3.3 mA m^{-2} total anode surface (TAS) for ^{13}C-SMFCs and 17.6 ± 3.8 mA m^{-2} TAS for ^{12}C-SMFC). Power densities in this period were 2.6 ± 0.9 mW m^{-2} TAS and 2.1 ± 0.9 mW m^{-2} TAS for the labeled SMFCs and the unlabeled SMFCs, respectively. Open circuit potentials by the reactors in open circuit reached values of 950 mV.

From day 44 to 51 of operation, planted SMFCs and open circuit controls were pulse labeled with ^{13}C-CO_2 as part of the stable isotope probing experiment. Unlabeled controls (planted SMFC and open circuit controls) were treated equally but with unlabeled CO_2. After every pulse with CO_2, the concentration in the chamber decreased from 3000 part per million in volume (ppmV) to 200 ppmV indicating that plants were successfully fixing CO_2 through photosynthesis. Total methane concentrations increased linearly with similar methane production rates for all SMFC (4.4 ± 0.6 μM h^{-1} for labeled SMFCs and 3.5 ± 0.4 μM h^{-1} for unlabeled SMFCs) and open circuit controls (3.3 ± 0.7 μM h^{-1} for labeled OC controls, 3.4 ± 0.5 μM h^{-1} for unlabeled OC controls). The atom percentage of ^{13}C-CH_4 increased linearly both in pore water samples and chamber gas samples in pulse-labeled SMFCs and OC controls but not in ^{12}C-CO_2 controls (Fig. 2) and the results obtained were similar to those reported previously (Lu & Conrad, 2005).

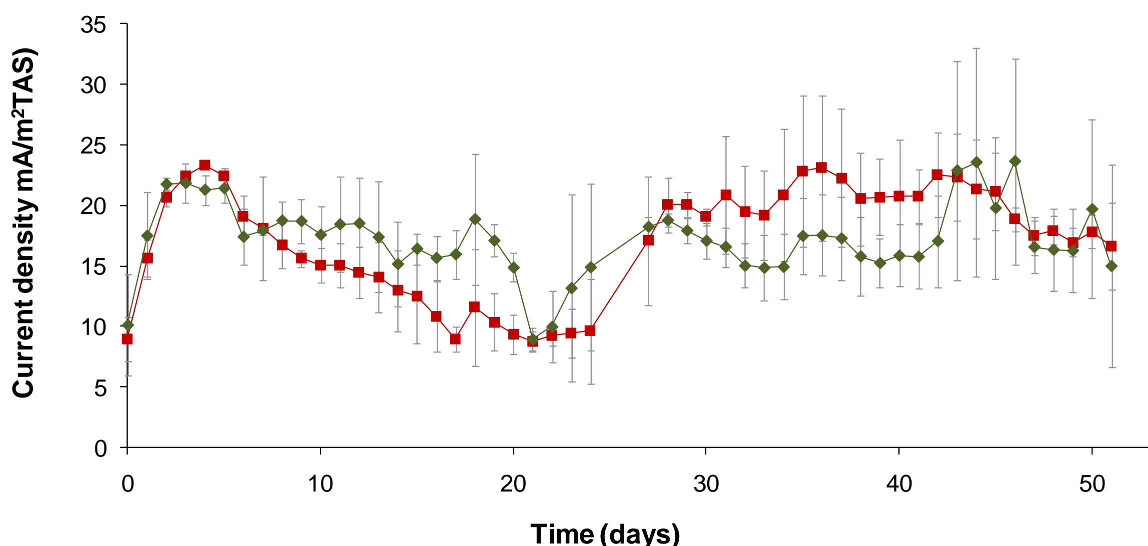

Figure 1 Current density production of planted sediment microbial fuel cells (SMFCs). The figure shows current density averages for planted sediment MFCs labeled with ^{13}C-CO_2 (n = 3; red square) and ^{12}C-CO_2 controls (n = 2; green diamonds) during operation time. Pulse-labeling was performed from day 44 to 51. Error bars indicate standard deviation between current density values of planted SMFCs.

Malate, formate, succinate, and acetate were detected in pore water samples from all setups (Fig. 3). Strong differences were detected in the acetate concentration; in OC controls acetate concentration was 10 times higher than in SMFCs (OC control: 40 ± 19 µM, SMFC: 4 ± 3 µM) (Fig. 3a). Malate concentrations decreased during the pulse labeling period, both in MFCs and open circuit controls, and were slightly higher in open circuit controls than in SMFCs (Fig. 3b). No significant differences in the concentration of succinate and formate were observed between SMFCs and open circuit controls and their concentrations remained constant throughout the pulse-labeling period (Fig. 3c and d).

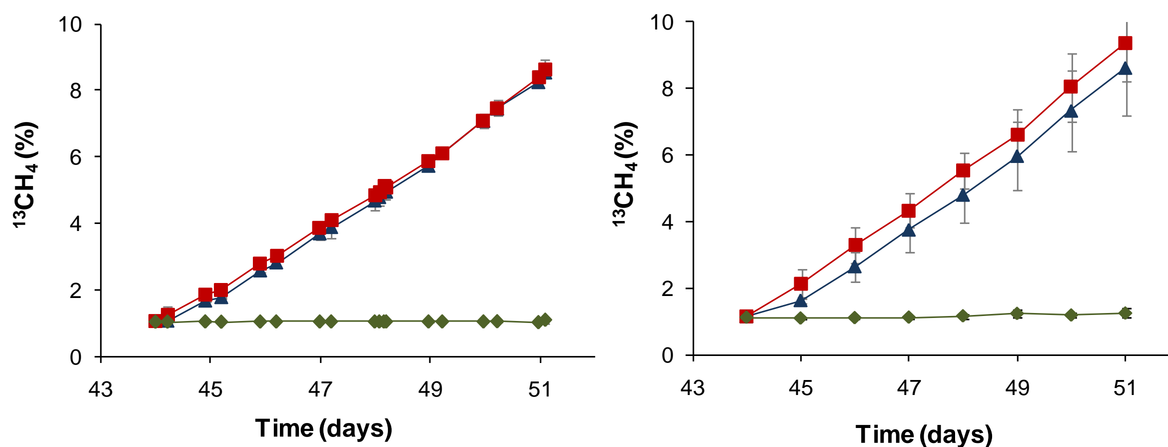

Figure 2 Increase of atomic percentage of $^{13}C\text{-}CH_4$ in a) chamber gas samples and b) pore water samples. The figure shows atomic percentage of $^{13}C\text{-}CH_4$ averages for planted SMFCs labeled with $^{13}C\text{-}CO_2$ (n = 3; red squares), open circuit controls labeled with $^{13}C\text{-}CO_2$ (n = 2; blue triangles), unlabeled controls (both MFCs and open circuit controls, n = 3; green diamonds). Error bars indicate standard deviations.

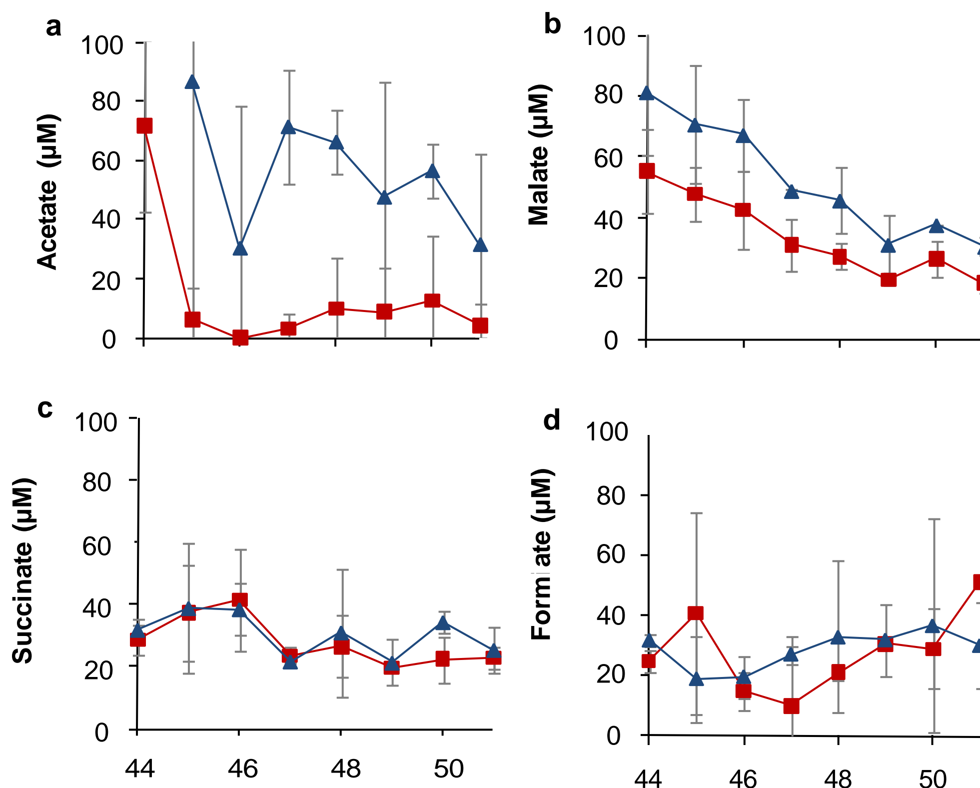

Figure 3 Pore water concentration (µM) of a) acetate, b) malate, c) succinate and d) formate in planted SMFC (Red squares) and open circuit controls (blue triangles) during stable isotope experiment.

High throughput sequencing and active bacterial community in compartments of planted SMFCs

After pulse labeling, anodes, bulk soil and roots were sampled for molecular analysis. 16S rRNA was extracted and "heavy" and "light" molecules separated by isopycnic centrifugation. 10 samples (including "heavy" and "light" fractions as well as SMFC bulk soil and root samples; Table S1) were analyzed by 454-pyroseqeuncing and a total of 158180 16S rRNA sequences were analyzed (Table 1). The number of sequences per sample varied strongly with the lowest value being 653 for MFC12CH and the highest 43451 for OC13CH. Quality filtering removed an average of 19% of sequences (see Table 1 for details of numbers) and the average length of the sequences was 395 ± 75 bp following the sequence quality filtering steps. The RDP- classifier assigns taxonomies

down to genus level accompanied with bootstrap-like confidence values. In previous reports ≥50% bootstrap values were recommended; lower bootstrap support than this resulted in very poor recovery (Liu*, et al.*, 2008, Claesson*, et al.*, 2009). For the assignment of taxonomy we used 60% as bootstrap threshold. Complete linkage clustering of the sequencing was performed and used to calculate Chao1 richness estimator, rarefaction curves and Shannon diversity index (Table1). To measure how phylotype richness in the different compartments of an SMFC samples varied (anode, soil, roots and anode from OC control), we calculated rarefaction curves at 95% similarity level (Fig. S1). Chao1 richness estimations and rarefaction curves indicated that even at this high level of sequencing it is evident that additional sampling increases the number of phylotypes detected. Community diversity, as reflected by the Shannon index, was highest in the anode of the OC control and lowest in the SMFC root sample, and is per definition generally correlated positively with the number of unique phylotypes and/or with greater community evenness. High evenness ($0<E<1$) indicates less variation in the relative abundance of phylotypes, i.e. the number of reads per phylotypes in this case. The SMFC root sample was less diverse and less even than the anode and soil samples (Table 1).

Table 1 Statistical characterization of 454-Pyrosequencing. Clusters, richness, diversity and evenness indexes were obtained using the RDP pipeline.

Datasets	Untrimmed reads	Trimmed reads	Clusters	Chao1 richness estimation	Chao1-LCI95	Chao1-UCI96	Shannon diversity	Species evenness
MFCH13C	18287	14974	3570	4.5	4.4	4.6	7.3	0.889
MFCL13C	2136	1796	764	1.2	1.1	1.3	6.1	0.925
MFCH12C	794	653	342	0.6	4.9	6.6	5.5	0.952
OCH13C	54569	43451	8278	9.8	9.7	9.9	7.9	0.873
OCL13C	8233	6611	2304	3.2	3.1	3.3	7.1	0.921
OCH12C	18200	14789	4018	5.3	5.2	5.5	7.5	0.904
MFCL12C	39628	32536	6279	7.5	7.3	7.6	7.7	0.880
OCL12C	37469	29765	6406	7.7	7.6	7.8	7.8	0.889
CC4soil	10526	8490	2731	3.7	3.6	3.8	7.3	0.923
CC4root	6293	5115	1278	1.8	1.7	1.9	6.2	0.865

Bacterial community compositions, assessed by 454-Pyrosequencing, of SMFC anode, SMFC bulk soil, SMFC roots and OC control anode differed. Sequences were grouped into 22 phyla and 16 phyla were detected in all four samples. Actinobacteria, Firmicutes, Proteobacteria and Chloroflexi were the most active phyla with more than 5% sequence abundance in all samples (Fig. 4). The most active phylum in the root sample was Proteobacteria (57%) followed by the planted SMFC (44%), OC control (31%) and bulk soil sample (28%). δ-Proteobacteria were highly active on the anode of SMFC comprising 70% of the Proteobacteria followed by β-Proteobacteria (14%). The root sample had the highest amount of sequences affiliated to β-Proteobacteria which comprised 30% of all Proteobacteria indicating high activity of this phylum on the root. However, the most active Proteobacteria on the root sample were δ-Proteobacteria comprising almost 50% of all Proteobacteria. The root sample also had the highest amount of sequences affiliated to α-Proteobacteria compared to bulk soil or anode samples (Fig. 4). On the planted SMFC, the most active δ-Proteobacteria were *Geobacter* spp. comprising 42% of all δ-Proteobacteria sequences (13% of the total bacterial community) followed by *Anaeromyxobacter* (17% of all δ-Proteobacteria) (Fig. 5c and Fig. S2). *Anaeromyxobacter* spp. comprised more than 50% of the total δ-Proteobacteria on rice roots and was the most active bacteria on the rice root sample (14% of the total bacterial community) (Fig 5c and Fig S2). *Anaeromyxobacter* spp. were also the most active bacteria on the open circuit anode almost reaching 6% of the bacterial community. Within β-Proteobacteria, 14% of unclassified Oxalobacteraceae sequences were detected on anodes from the planted SMFC while less than 2% was found on the other samples (Fig. 5d and Fig. S2). Similarly, 8% of sequences from the SMFC anode grouped within unclassified Comamonadaceae while less than 3% were detected in the other samples. On rice roots the predominant β-Proteobacteria belonged to unclassified Burkholderiales, *Methylibium* spp., *Pelomonas* spp., and unclassified Rhodocyclaeceae indicating activity of these populations on rice roots. On bulk soil and open circuit control samples unclassified Rhodocyclaceae and *Methyloversatilis* spp. were active (Fig. 5d and Fig S2).

The lowest amount of Chloroflexi was found on the root sample (5 %) while bulk soil, SMFC anode and OC anode samples had similar amounts (10%, 8%, 8%, respectively). Most of the Chloroflexi sequences (95%: SMFC anode, 95%: SMFC soil,

97%: SMFC root, 94%: OC anode) were classified within the Anaerolineae lineage and was the most active phylogenetic group found in bulk soil sample (6% of all sequences) (Fig. 4 and Fig S2).

Low amount of sequences from the SMFC anode and root samples grouped within the phylum Firmicutes (7% and 5%, respectively) compared to SMFC bulk soil and OC anode samples (17% and 18%, respectively) (Fig. 4). *Bacillus* spp. was the most active Firmicutes in the SMFC anode however, also in the OC anode. In contrast, *Clostridium* spp. predominated SMFC bulk soil and root samples and was the second most active phylogenetic group in bulk soil after Anaerolineae (Fig. 5a and Fig S2). The abundance of Actinobacteria was similar in all samples but the composition of Actinobacteria on the SMFC rice root was different from the other samples with high abundance of unclassified Actinomycetales, unclassified Kineosporiaceae, *Kinoecoccus* spp. and *Marmoricola* spp. were highly active in all samples except the root sample where it only comprised 2% of the actinobacterial community (Fig 5b and Fig S2).

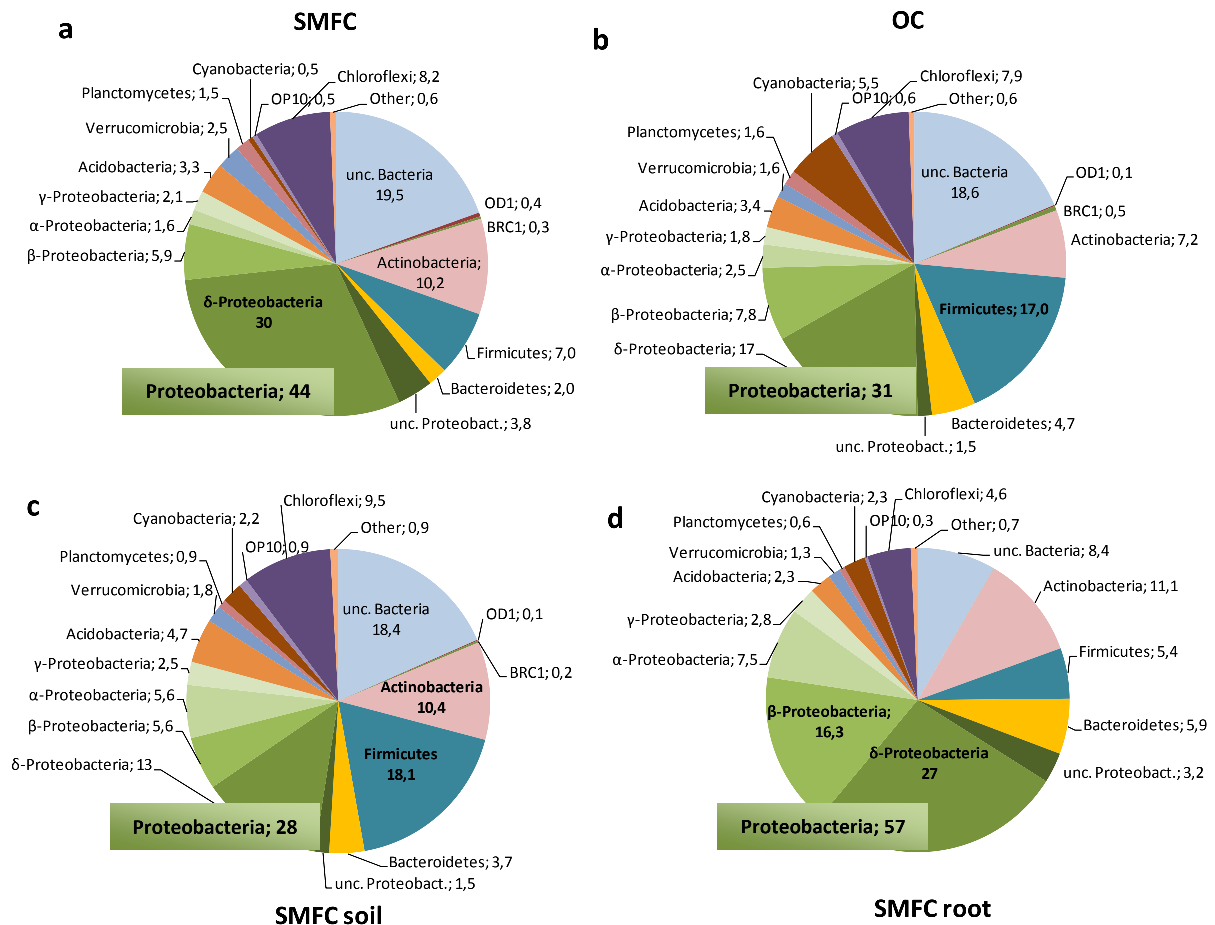

Figure 4 Bacterial diversity on anodes from a) planted sediment microbial fuel cell (SMFC) b) open circuit control (OC) c) SMFC bulk soil (SMFC soil) and d) SMFC rice roots (SMFC root) at a phylum level. The Phylum Proteobacteria is subdivided into Proteobacteria classes. The number next to the phylum refers to the amount of sequences in percentage.

Identification of labeled bacteria by T-RFLP analysis and 454-pyrosequencing of 16S rRNA density isopycnic centrifugation gradient fractions

The anode microbial community of planted SMFC was dominated by *Geobacter* spp., *Anaeromyxobacter* spp. and Anaerolineae and when comparing to the open circuit control, it becomes evident that *Geobacter* spp. play a relevant role in current production. However, whether root exudates are directly used for current production has still to be elucidated. To identify which anode bacteria degrade root exudates, a RNA-

stable isotope probing (RNA-SIP) experiment, where ^{13}C-CO_2 was added to planted SMFC. The fractions from the isopycnic gradient were analyzed by two methods, T-RFLP and 454- pyrosequencing. T-RFLP profiles for all gradient fractions with densities ranging from 1.772 g ml^{-1} to 1.826 g ml^{-1} from SMFC and open circuit control samples, both labeled and unlabeled, were analyzed. By comparing T-RFs relative abundance in "heavy" and "light" gradient fractions we could determine which sequences were labeled. Two 16S rRNA sequences with T-RFs of 510 bp and 214 bp were labeled only in planted SMFCs but not in unlabeled controls or labeled open circuit controls (Fig. 6). This would indicate that bacteria presenting these T-RFs were actively degrading root exudates probably, with concomitant current production. Four 16S rRNA sequences with T-RFs of 114 bp, 120 bp, 136 bp and 489 bp were labeled both in the pulse-labeled planted SMFCs and open circuit controls which indicates that bacteria with these T-RFs might be actively degrading root exudates. Two 16S rRNA sequences with T-RFs of 147 bp and 431 bp were labeled only in the pulse labeled open circuit control (Fig. 6) which indicated that bacteria with these T-RFs were able to degrade root exudates only when current was not produced.

Bacterial communities of "heavy" and "light" gradient fractions from the different SIP experiments were analyzed by 454-pyrosequencing. 454-pyrosequencing also allowed us to assign the T-RFs detected as the 16S rRNA region used for 454-pyrosequencing was included in the region used for T-RFLP. For assignment of predominant T-RFs, phylogenetic trees with representative sequences (>450bp) from the 60 most abundant clusters obtained by complete linkage clustering were constructed using ARB software and *in silico* restriction T-RFs were determined from the alignments. T-RFs of 214 bp and 510 bp which corresponded to bacteria labeled only in the planted SMFC were assigned to *Dehalococcoide*s spp. and *Anaerolineae* spp., respectively. According to the T-RFLP analysis, four populations were labeled both in the open circuit control and the planted SMFC.

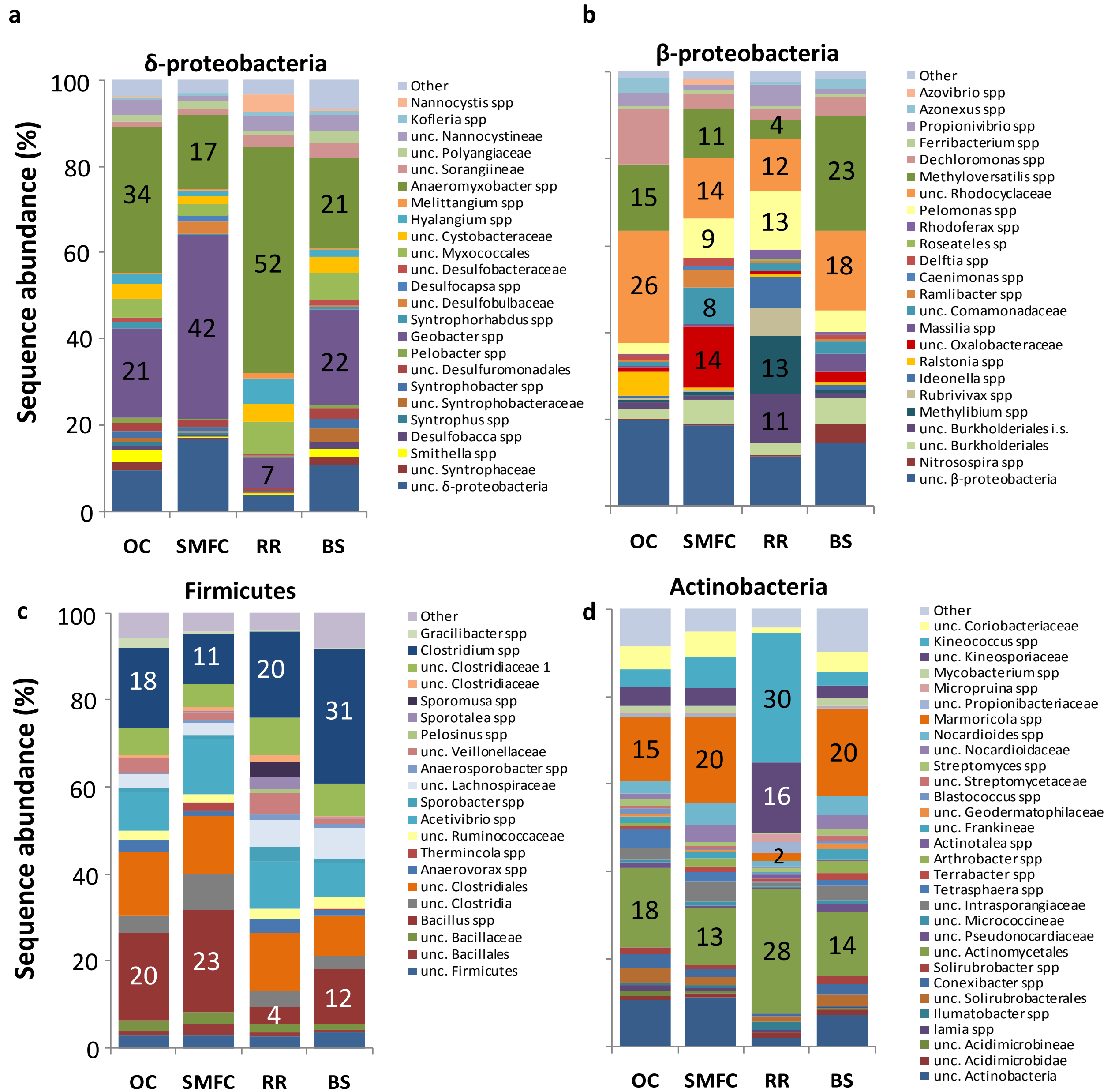

Figure 5 Bacterial composition of open circuit control (OC) planted SMFC (SMFC), SMFC rice root (RR) and SMFC bulk soil (BS) within the Proteobacteria classes a) δ-Proteobacteria b) β-Proteobacteria and the Phyla c) Firmicutes and d) Actinobacteria. The numbers indicate the percentage related to the respective phylum or class.

The T-RFs of these bacteria were assigned to *Anaerolineae* spp. (114-bp and 120-bp), unclassified Gallionelaceae (120-bp), unclassified Rhodocyclaceae (489-bp), unclassified Comamonadaceae (489-bp) and unclassified Kineosporiaceae (136-bp).

Regarding the T-RFs only increasing in the open circuit control, 431-bp was assigned to *Dechloromonas* spp. and unclassified Oxalobacteraceae and 147-bp to *Bacillus* spp.

When comparing the 454-pyroseqeuncing data of “heavy” and light” fractions of labeled SMFC and OC controls with unlabeled controls we were able to further identify bacteria actively using root exudates on SMFC anodes and OC controls. Phylogenetic classification of the sequences by RDP classifier revealed that β-Proteobacteria and Chloroflexi became labeled in planted SMFCs indicating that bacteria from these phyla were actively degrading root exudates in current producing SMFCs (Table 2). In the OC control, only sequences belonging to β-Proteobacteria were found labeled. Even though the most abundant taxonomic group in the planted SMFC were δ-Proteobacteria, no differences in sequence abundance between “heavy” and “light” fractions were detected. When analyzing at lower phylogenetic levels, sequences belonging to unclassified Oxalobacteraceae and *Acidovorax* (β-Proteobacteria) and Anaerolineae (Chloroflexi) were detected labeled in the planted SMFC (Fig. 7a) while in the open circuit control *Dechloromonas* (β-Proteobacteria) was labeled (Fig. 7b). However, some Actinobacteria were also found labeled; *Kineococcus* and unclassified Kineosporiaceae were labeled in the OC control (Fig. 7b) while *Marmoricola* spp. were labeled in the planted SMFC.

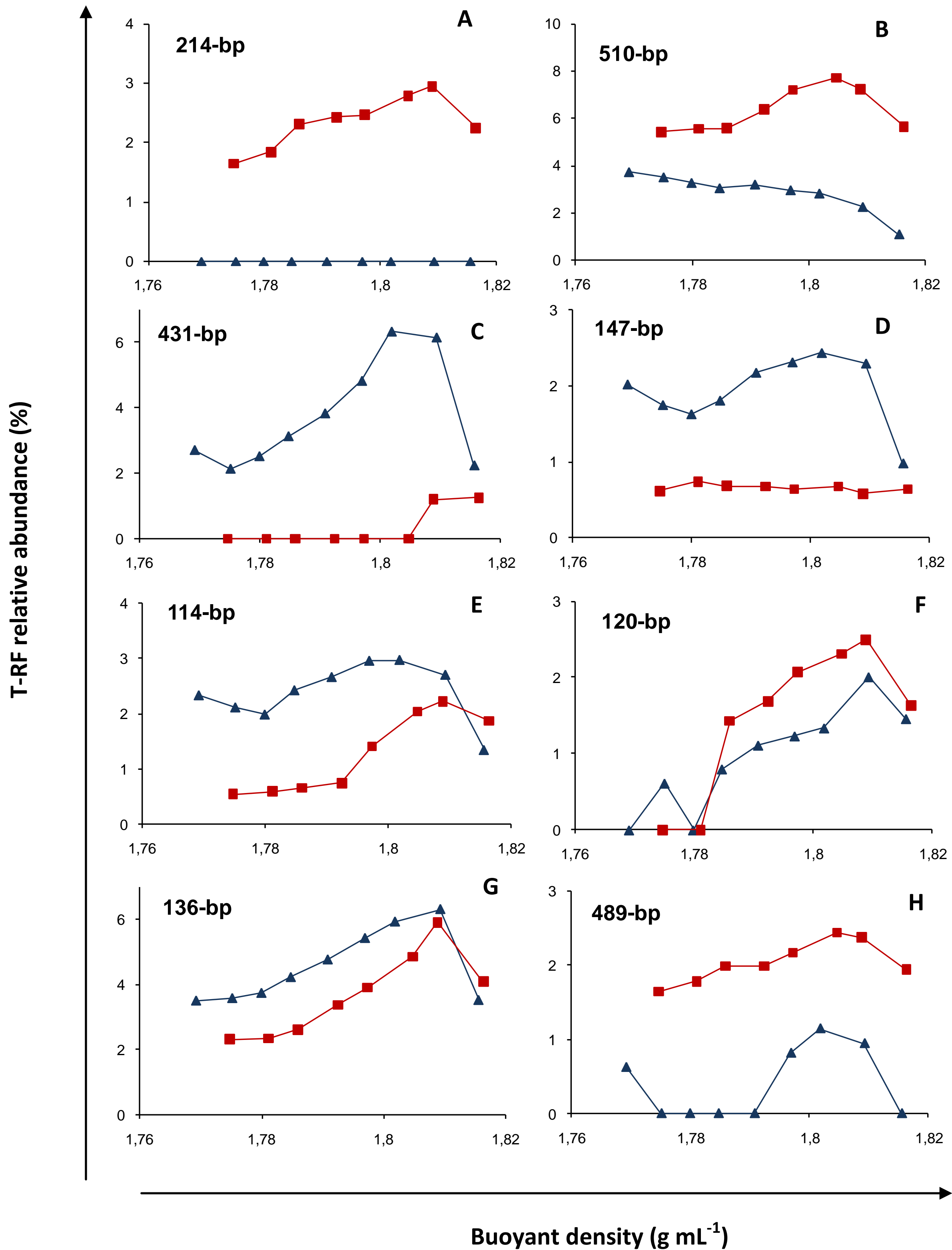

Figure 6 T-RFs relative abundance in gradient fractions of ^{13}C-labled SMFC (red) and OC control (blue). Panels A and B: T-RFs labeled only in SMFCs; panels C and D: T-RFs labeled only in OC controls; Panels E, F, G and H: T-RFs labeled both in SMFCs and OC controls. Size of TRF is shown in each graph in bp.

Table 2. Taxonomic groups present on "heavy" and "light" fractions of planted sediment microbial fuel cells (SMFC) and open circuit controls (OC) determined by analysis of sequences from 454-pyrosequencing using RDP classifier. The classes of Proteobacteria are shown. Labeled taxonomic groups are marked in red.

	^{13}C-SMFC		^{12}C-SMFC		^{13}C-OC		^{12}C-OC	
Taxonomic group	Heavy	Light	Heavy	Light	Heavy	Light	Heavy	Light
Unc. Bacteria	19.8	18.5	21.6	19.5	19.7	19.1	20.8	18.6
OD1	0.2	0.5	0.3	0.4	0.1	0.1	0.1	0.1
BRC1	0.3	0.2	0.3	0.3	0.6	0.6	0.5	0.5
Actinobacteria	9.9	14.4	13.5	10.2	13.6	9.5	10.6	7.2
Firmicutes	5.4	6.2	4.6	7.0	14.8	18.9	15.2	17.0
Spirochaetes	0.1	0.2	0.2	0.1	0.3	0.3	0.2	0.2
Bacteroidetes	2.1	2.5	1.2	2.0	4.2	3.5	4.1	4.7
Proteobacteria	30.0	30.8	30.8	32.3	17.6	19.3	17.1	19.0
Unc. Proteobacteria	3.2	4.3	3.4	3.8	1.5	1.2	1.5	1.5
α-Proteobacteria	1.4	1.4	0.9	1.6	2.2	2.1	2.1	2.5
β-Proteobacteria	**9.6**	**4.6**	6.4	5.9	**9.0**	**6.2**	6.7	7.8
γ-Proteobacteria	3.0	2.0	2.1	2.1	2.2	1.7	1.8	1.8
δ-Proteobacteria	27.0	28.7	28.6	30.2	15.3	17.6	15.3	17.1
ε-Proteobacteria	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Gemmatimonadetes	0.1	0.3	0.2	0.1	0.2	0.1	0.1	0.1
Acidobacteria	2.2	3.5	4.1	3.3	2.3	3.8	3.2	3.4
Verrucomicrobia	2.5	2.5	1.8	2.5	2.1	2.8	1.4	1.6
WS3	0.4	0.1	0.2	0.2	0.1	0.1	0.1	0.1
Planctomycetes	1.8	1.6	2.9	1.5	2.8	1.6	2.9	1.6
Cyanobacteria	0.7	0.3	1.7	0.5	1.5	1.1	5.3	5.5
OP10	0.7	0.3	0.2	0.5	0.6	0.7	0.6	0.6
Chloroflexi	**9.4**	**7.3**	5.4	8.2	6.5	8.7	7.3	7.9
Other	0.5	0.6	0.9	0.4	0.5	0.5	0.5	0.6

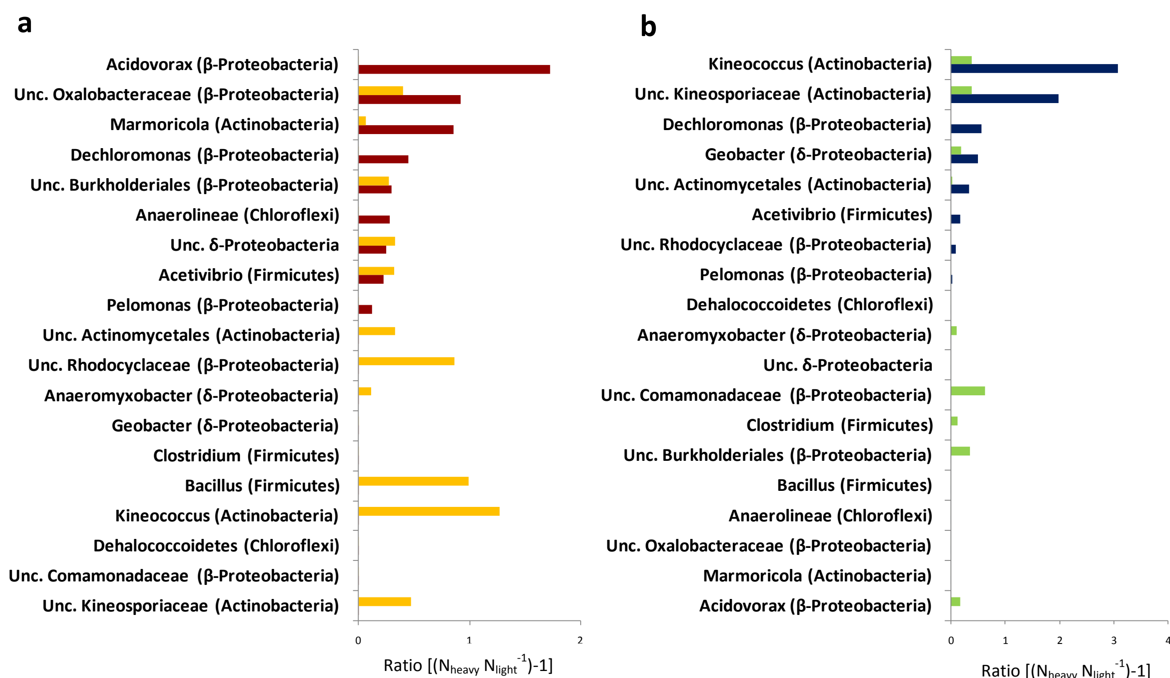

Figure 7 Labeled phylogenetic groups in a) planted sediment microbial fuel cells (red) and b) in open circuit controls (blue). Unlabeled controls are included in each graph (yellow and green). Ratio was calculated as the relative abundance in "heavy" fractions (N_{heavy}) divided relative abundance in "light" fraction (N_{light}) as follows: [(N_{heavy} N_{light}^{-1})-1]. Negative values were not included in the graphs.

5.5 Discussion

Bacterial community structure in planted SMFC

Flooded rice paddy soils can be considered as a system with three compartments; oxic surface soil, anoxic bulk soil, and rhizosphere, characterized by different physiochemical conditions. (Liesack, et al., 2000). Moreover, owing to leakage of O_2 and organic substances from roots, the rice roots and the rhizosphere provide niches for diverse organisms performing various biogeochemical processes (Lu, *et al.*, 2006). In planted SMFCs, the anode could be considered as an additional compartment with different physiochemical characteristics. Here, we studied the bacterial community structure in three different compartments of a planted SMFC: bulk soil, anode and roots, by 454-pyrosequencing. If we consider the bulk soil as our microbial diversity "reservoir" where a more even community was observed, clearly certain bacterial groups became active on

anode and root samples. Rice roots were dominated by *Anaeromyxobacter* spp. within δ-Proteobacteria and β-Proteobacteria probably due to the type of environmental conditions developed around roots. For example, the release of oxygen through the roots enhances the oxidation of iron(II) to iron(III). Some *Anaeromyxobacter* spp. are known iron reducers and their function on roots might be related to the reduction of the iron (III) produced in the vicinity of rice roots (Treude*, et al.*, 2003). The role of β-Proteobacteria on SMFC roots might be related to rice root exudate degradation as β-Proteobacteria have found previously as the active population on rice roots (Lu*, et al.*, 2004).

On anodes, a selection of electrogenic bacteria occurred and *Geobacter* became the predominant microorganisms. Comparing with open circuit controls, *Geobacter* are responsible for current generation. However, high abundance of *Anaeromyxobacter* was also detected which suggest that these iron reducing bacteria might also be able to reduce anodes. The betaproteobacterial community composition on anodes of planted SMFCs differed from the root sample. One explanation could be that the β-Proteobacteria developed on the anode, for example Oxalobacteraceae and unclassified Comamonadaceae might be able to transfer electrons to the anode. A second possibility could be that the presence of an anode changes the microenvironment and/or the possible metabolic pathways, affecting the bacterial community compositions. Firmicutes was predominant in the bulk soil sample, in particular, *Clostridium* which might be responsible for the degradation of polymers, such as xylan, pectin, and cellulose, abundant in bulk soil (Liesack*, et al.*, 2000).

β-Proteobacteria and Chloroflexi actively degrade root exudates in anodes from SMFCs

The presence of rice plants in SMFCs increases current output and selects for a specific microbial community (De Schamphelaire*, et al.*, 2008, De Schamphelaire*, et al.*, 2010). This increase in current is probably due to the release of root exudates into the soil which increases the organic matter available for current production. However, it is not known whether root exudates are used directly to produce current in planted SMFCs. Here, we identified the bacteria actively assimilating root exudates on anodes of planted SMFCs by rRNA-SIP, T-RFLP and 454-pyrosequencing as belonging to β-Proteobacteria

and Anaerolineae. The main betaproteobacterial species labeled on the anode of the planted SMFC belonged to the family Oxalobacteraceae and the genus *Acidovorax*. *Oxalobacter* spp. have been found in soils and sediments and are able to degrade oxalate into formate and CO_2 anaerobically (Dehning and Schink, 1989; Cornick and Allison, 1996). Oxalate has been found to be an important compound from root exudates and its chelating capacity benefits the plant reducing the concentration of metals (Steven*, et al.*, 2007). Our results would suggest that unclassified Oxalobacteraceae might play a role in the degradation of root exudates coupled to current production also supported by the increase of the activity of unclassified Oxalobacteraceae on the anode of the planted SMFC compared to roots, bulk soil and open circuit control anode. *Acidovorax* have been previously found abundant on anodes from two chamber MFCs inoculated with river sediment (Phung*, et al.*, 2004) and in single chamber MFCs (Borole*, et al.*, 2009, Lefebvre*, et al.*, 2010) which suggest that *Acidovorax* might be degrading root exudates with concomitant electron transfer to the anode. *Dechloromonas* spp., unclassified Rhodocyclaceae, unclassified Comamonadaceae were found labeled in the planted SMFC as well as on the anode of the OC control. Lu *et al* (Lu*, et al.*, 2006) detected labeled Burkholderiaceae (related to *Paucimonas lemoignei*) within the β-Proteobacteria as well as *Azospirillum* related sequences within α-Proteobacteria as the active root exudate degraders in the rhizospheric soil in a similar SIP experiment. A different plant variety was used in this study which might produce a different root exudate pattern selecting for different active populations.

Anaerolineae have been found to be part of rice field soil microbial communities (Rui*, et al.*, 2009) as well as on anodes of planted SMFC (De Schamphelaire*, et al.*, 2010). Here, Anaerolineae became labeled indicating that these species might use root exudates. However, T-RFLP showed that Anaerolineae species were labeled both in SMFCs as in OC controls and therefore, it is still not clear whether these species also contribute to current production.

5.6 Conclusions

Bacterial communities on different compartments of SMFCs fueled by rice root exudates analyzed by 454-pyrosequencing differed with a selection of δ-Proteobacteria and β-

Proteobacteria on roots and δ-Proteobacteria on anodes. *Geobacter* and *Anaeromyxobacter* predominated on the anodes and are probably playing a role in current production in planted SMFCs. Probably, the microenvironments encountered around the root system and the anode determined the bacterial community compositions. Stable isotope probing with $^{13}C\text{-}CO_2$ combined with T-RFLP and 454-pyrosequencing, allowed to identify the bacteria actively degrading root exudates as belonging to β-Proteobacteria and Anaerolineae. Moreover, the labeled bacteria differed in the SMFC compared with the OC control which could indicate that some bacteria are coupling current production with root exudate degradation. Most likely, a microbial "food chain" is responsible for current generation in these systems where close interaction between root exudate degrading bacteria and anode reducing bacteria is necessary.

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5.8 Suplementary data

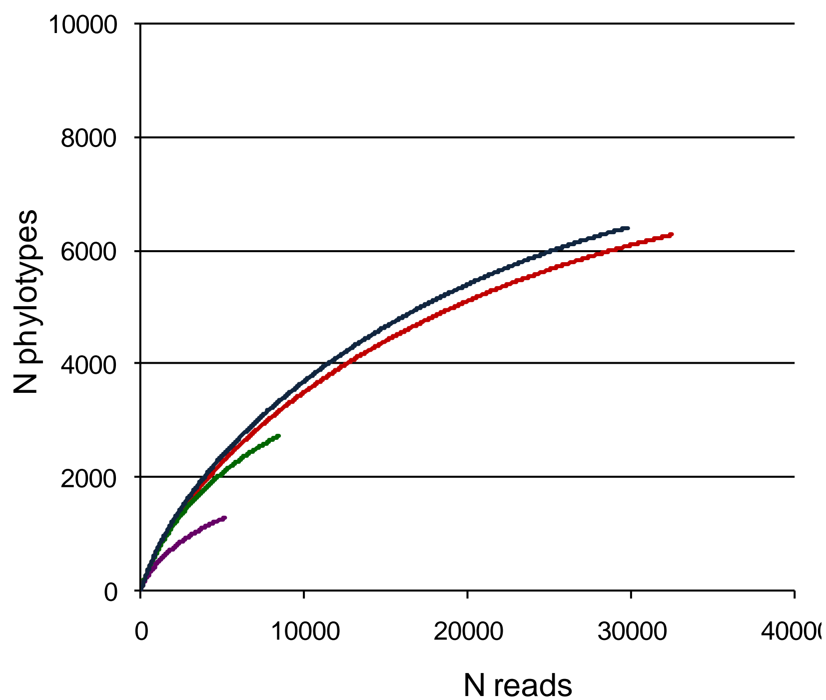

Figure S1 Rarefaction curve at 95 % similarity for SMFC anode (red), SMFC bulk soil (green), SMFC root (violet), OC control anode (blue).

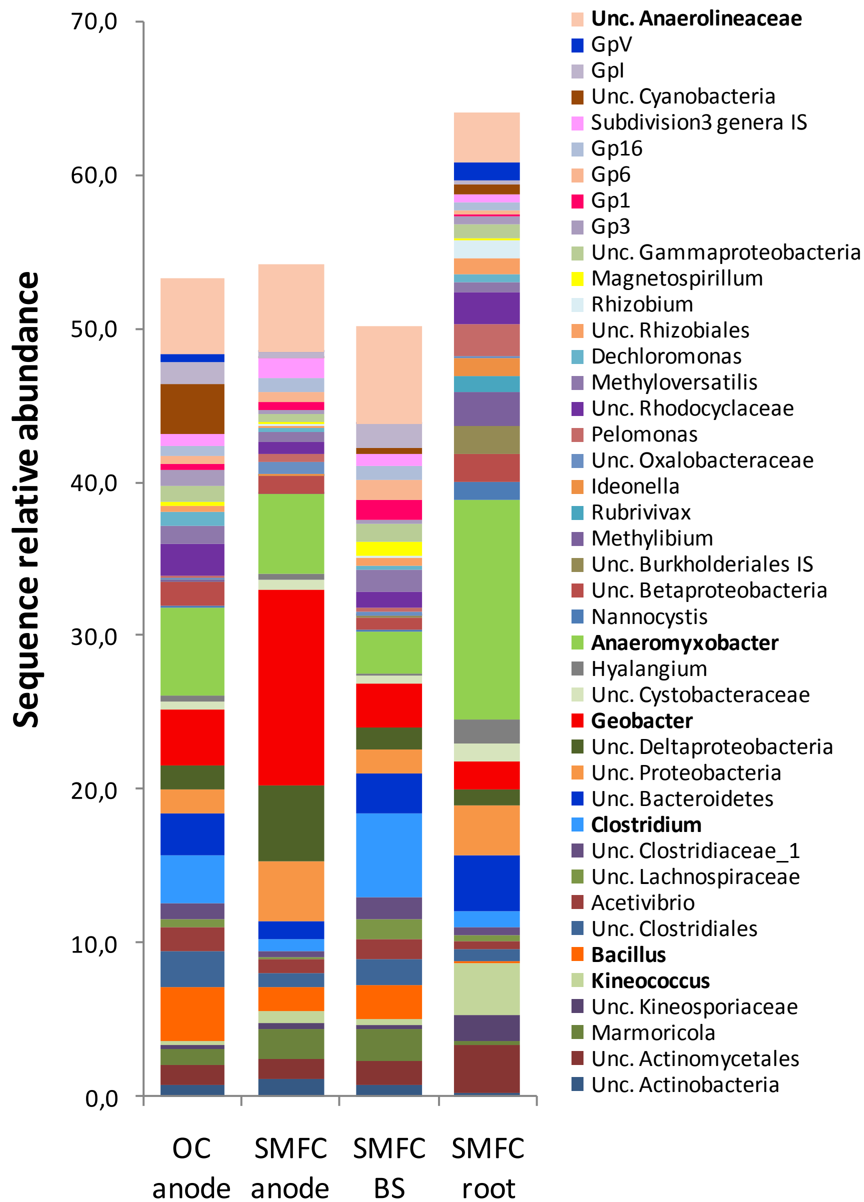

Figure S2 Predominant phylogenetic groups found on SMFC anodes, SMFC bulk soil, SMFC roots, OC control anodes. Only groups with sequence relative abundance higher than 1% in at least one sample were included. Unclassified Bacteria were not included in the graph.

Table S1. Nomenclature of samples analyzed by 454-pyrosequencing.

Sample	Density (mg ml^{-1})	Description
OCH12C	1.784	"Heavy" gradient fraction of open circuit control labeled with 12C
OCL12C	1.806	"Light" gradient fraction of open circuit control labeled with 12C
OCH13C	1.811	"Heavy" gradient fraction of open circuit control labeled with 13C
OCL13C	1.784	"Light" gradient fraction of open circuit control labeled with 13C
MFCL12C	1.808	"Light" gradient fraction of MFC labeled with 12C
MFCH12C	1.786	"Heavy" gradient fraction of MFC labeled with 12C
MFCL13C	1.808	"Light" gradient fraction of MFC labeled with 13C
MFCH13C	1.784	"Heavy" gradient fraction of MFC labeled with 13C

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Chapter 6

Taming Methane Emissions from Rice Field Soil with Microbial Fuel Cells

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Author Contributions

AC and MWF designed this study and were responsible for preparing the manuscript. BB constructed, operated and monitored the MFCs, performed methane measurements and sampled the anodes. BB and AC performed RNA extractions and T-RFLP analysis of anode samples. BB and AC performed data analysis.

6.1 Abstract

Microbial fuel cells (MFCs) are devices in which microorganisms convert the chemical energy present in organic matter into electrical energy. Electrogenic bacteria have been shown to produce electrical current from specific substrates as well as from diverse wastewaters. However, applications of microbial fuel cells other than current production have not been thoroughly explored. Here, we show that methane production from rice paddy soils can be regulated by using a sediment microbial fuel cell. Rice paddies are a major source of the greenhouse gas methane contributing up to 15% to the global atmospheric emission budget. In rice paddy microcosms, methane emission was reduced up to 47 % when the anode of a MFC was available as electron acceptor. Remarkably, the methane suppression observed was ~100-fold more effective than electricity production expected based on methane carbon oxidation stoichiometry. Thus, a biogeochemical engineering application of MFCs, such as mitigation of methane emission, might be a much more pertinent role for MFCs than harvesting electricity. Our findings open the door for applications of microbial fuel cells to control biogeochemical processes that have a negative impact on climate (e.g., trace gas emissions).

6.2 Introduction

The depletion of fossil fuels and environmental pollution from fossil fuel combustion is driving the search for renewable energy alternatives. Microbial fuel cells (MFCs) are new biotechnological devices for green energy production based on current generation from microbial activity. In MFCs, electrogenic microorganisms are able to transfer electrons from the oxidation of organic matter to the anode of an MFC; concomitantly, electrical current is generated when anode derived electrons are transferred to the cathode, and re-oxidized chemically with oxygen as terminal acceptor (Logan*, et al.*, 2006, Lovley, 2006). In order to improve power output and make current generation with MFCs economically feasible various MFC designs, anode materials, operation conditions, substrates, and microorganisms as catalysts have been tested (Rabaey & Verstraete, 2005, Logan*, et al.*, 2006, Du*, et al.*, 2007, Pant*, et al.*, 2010) and in the past years, power output of MFCs have been successfully increased. MFCs have a bright future as

power sources in wastewater treatment plants due to the ability to recover energy from wastewater directly in the form of electricity (Erable*, et al.*, 2010). Moreover, the possibility to even harness power from organic matter in the sea floor using sediment microbial fuel cells (SMFCs) has been demonstrated (Tender*, et al.*, 2002) and in 2008 the application of SMFC as power supply for a meteorological buoy was reported (Tender*, et al.*, 2008). This particular type of MFC consists of an anode buried in anoxic sediment and a cathode positioned in the oxic overlying waterbody (Tender*, et al.*, 2002, Lovley, 2006).. More recently, a SMFC was employed to produce electrical energy from rhizodeposits of rice plants, e.g. organic exudates from roots, and thus, ultimately using photosynthetically fixed carbon (De Schamphelaire*, et al.*, 2008, Kaku*, et al.*, 2008). Combining current generation by MFC with other alternative applications is an interesting option for enhancing the application perspectives of MFCs. Controlling trace gas emissions, combined with current generation in sediment MFCs, has not been identified up to date as an application of SMFCs.

Methane contributes up to 20 % of the total radiative forcing of anthropogenically produced greenhouse gases, which may result in an increase of the global surface temperature of 2–4°C within the next 100 years (IPCC-2007). The methanogenic Archaea in rice field soil produce approximately 15 % of the global CH_4 emissions (Lelieveld*, et al.*, 1998) and rice paddies are therefore a major source of atmospheric methane, with an estimated 25-60 Tg a^{-1} (IPCC-2007). Water management and fertilization, for instance with iron (III) oxides, have been suggested and tested for reducing methane emission from rice paddies (Conrad, 2002); these mitigation strategies alternate redox regimes (oxic-anoxic), which regenerates or replenishes alternative electron acceptors such as ferric iron oxides (Conrad, 2002). Under these conditions, methanogens can be out-competed for common electron donors by ferric iron-reducing bacteria (Achtnich*, et al.*, 1995). However, iron fertilization is expensive and high iron oxide levels may reduce crop yield due to iron toxicity (Jackel & Schnell, 2000).

Here, we show that SMFCs can be used to reduce methane emission from paddy soils, while generating electrical current as a by-product. Our data show that microbial fuel cell technology has currently a larger potential for controlling trace gas emissions than for electricity production.

6.3 Material and Methods

Sediment microbial fuel cells (SMFC)

The SMFCs were constructed in 500 mL Bottles (Schott, Mainz) using 350 g of rice field soil which was flooded leaving a 4 cm water layer. The rice field soil was sampled from the research institute "Instituto Sperimentale per la Cerealicoltura" in Vercelli, Italy, air dried and stored at room temperature. Before use the soil was homogenized and sieved through a 2 mm sieve. In each sediment MFC an anode was placed in the soil matrix, three pore water samplers (RHIZON FLEX Soil Moisture Samplers, Eijkelkamp, Giesbeek, Niederlande) and a cathode (62 cm^2) in the overlying water layer which was aerated with an aquarium pump. The anodes and cathodes were made of carbon felt (Alfa Aesar, Ward Hill, USA) and the connection was made through a graphite rod (5mm diameter, Thielmann Graphite GmbH, Grolsheim, Germany).

Three series of SMFC (A, B and C) were constructed in duplicates (A and B) and triplicates (C) and differed in anode size, time of operation and external resistance. Series A and B were operated for 72 days and the resistance was 470 Ω until day 24 and 100 Ω from day 24 to day 72. The total anode surface (TAS) for series A was 540 cm^2 while series B had half the TAS (270 cm^2). Series C was operated for 35 days, had an anode size of 270 cm^2 and an external resistance of 100 Ω. Unconnected sediment MFCs were constructed as controls in duplicates and triplicates exactly like the connected sediment MFCs. The Potential (mV) was measured every 15 minutes with a Datalogger (Agilent 34970A, Agilent Technologies, Böblingen). Current and Power densities were calculated as reported previously (Logan, *et al.*, 2006).

The incubations were made in the dark and at 25°C. 5 mL pore water samples were taken in 10 mL vials. Acetate concentration in the pore water was measured by high-pressure liquid chromatography (Krumbock & Conrad, 1991). Methane and CO_2 were measured in the vial headspace by gas chromatography (Roy, *et al.*, 1997). The 13C atoms percent of CH_4 was determined by GC-isotope ratio mass spectrometry (Conrad, *et al.*, 2000). At the end of each experiment, anodes were sampled and stored at -80°C for further molecular studies.

Molecular analysis of the archaeal community

4 parallel RNA extractions with 0.5g of anode material each were performed using a bed beating protocol as described previously (Lueders*, et al.*, 2004). T-RFLP analysis was performed according to Egert *et al* (2003). Briefly, 16S rRNA was used as a template for reverse transcription – PCR (RT-PCR) using a single step RT-PCR system (Access Quick, Promega, Mannheim, Germany). The primers Ar109f and FAM-Ar912r were used to specifically amplify Archaea. PCR products were cleaned up (GenElute™PCR Clean-Up Kit, Sigma-Aldrich) and ~100 ng were digested with the restriction enzyme *Taq*I (Promega). 1-2 µl of cleaned (SigmaSpin™ Post-Reaction Clean-Up Columns, Sigma-Aldrich) digestion product were mixed with 11 µl of Formamide (Hidi; Applera Deutschland GmbH, Darmstadt) and 0,3 µl Molecular weight marker (X-Rhodamine MapMarker® 1000, BioVentures, Murfreesboro, Tennessee, USA) and denaturized 3 minutes at 95°C. Electrophoresis was performed on an ABI PRISM 3130 Genetic Analyzer. T-RFLP electropherograms were analyzed with GeneMapper® Software 4.0 (Applied Biosystems). Tables were extracted for each sample with peak size *vs*. fluorescence intensity and TRFs that differed by ±1 bp in different profiles were considered as identical in order to compare the T-RFLP profiles between different samples. The peak heights were standardized to the minimum sample according to Dunbar *et al.* (2001).The relative abundance of each T-RF within a given T-RFLP pattern was calculated as the peak height of the respective T-RF divided by the total peak height of all T-RFs detected within a fragment length range between 50 and 900 bp. Principal component analysis was performed using PAST in order to identify the main components determining the clustering of the T-RFLP samples from MFCs and controls (Fig. S3).

Cloning of the 16S rRNA gene was performed using pGem T cloning kit from Promega according to the manufacturer's protocol. RT-PCR was performed as mentioned above using unlabeled primers. Random colonies were selected and 16S rRNA gene amplified with primers flanking the vector (M13F and M13R). PCR product size was verified by gel electrophoresis and forward and reverse sequencing was performed (Qiagen, Germany). Sequences analysis was performed by ARB parsimony tool and RDP classifier obtaining same result with both methods. *In silico* restriction fragment sizes (*in*

silico T-RFs) were obtained by manual search in ARB for the restriction site of the restriction enzyme *Taq*I (T|CGA).

6.4 Results and discussion

Average current densities (Table 1, Table S1) were calculated in stable current production periods and were well within the range of similar sediment microbial fuel cells (De Schamphelaire*, et al.*, 2008). Nevertheless, MFC-C had a higher current output, probably due to the use of lower external resistance (Hong*, et al.*, 2009). Due to the anaerobic degradation of organic matter, methane and carbon dioxide concentrations in pore water constantly increased over time in all SMFCs and open circuit controls (Fig. 1). However, methane production rates were considerably lower in SMFCs compared to the open circuit controls (Table 1). Microcosms with closed circuit MFCs reduced methane production up to 47%. Anode surface size strongly affected methane production and doubling anode surface reduced methane production by a factor of two (Table 1, Fig. 1), suggesting that methane production was dependent on anode surface of closed circuit MFCs. A comparable mitigation of methane production reduction from a flooded soil MFC system has not been reported before; rice field soil was merely used as an inoculum (1% w/v) for an H-type MFC, operated under well mixed conditions, and thus, not comparable to methane formation in a flooded soil (Ishii*, et al.*, 2008).

Table 1 Methane production rates and relative abundance of select methanogenic Archaea in sediment MFCs. Rates were determined from methane concentrations measured in pore water samples. Reduction of methane emission (%) was calculated taking the control as 100%. The relative abundance of the methanogenic Archaea *Methanosaeta* spp. was determined by T-RFLP fingerprinting from the relative abundance of T-RF 285 bp. MFC A: TAS = 540 cm^2, 470 Ω, 72 days; MFC B: TAS = 270 cm^2, 100 Ω, 35 days

Sediment MFC (TAS, cm^2)	Average current density (mA m^{-2}TAS)	Methane production rate (μM day^{-1})	Reduction of methane production rate (%)	Decrease of relative abundance (%) 285 bp T-RF *(Methanosaeta)*
MFC A (540)	4.6 ± 0.3[a]	113 ± 21	47	66 ± 10
Control A (540)		211 ± 21		
MFC B (270)	7.1 ± 0.5[a]	146 ± 7	24	79 ± 31
Control B (270)		192 ± 41		
MFC C (270)	42 ± 4[b]	166 ± 57	36	55 ± 12
Control C (270)		259 ± 66		

[a] Average calculated for operation days 23-70.
[b] Average calculated for operation days 17-35.

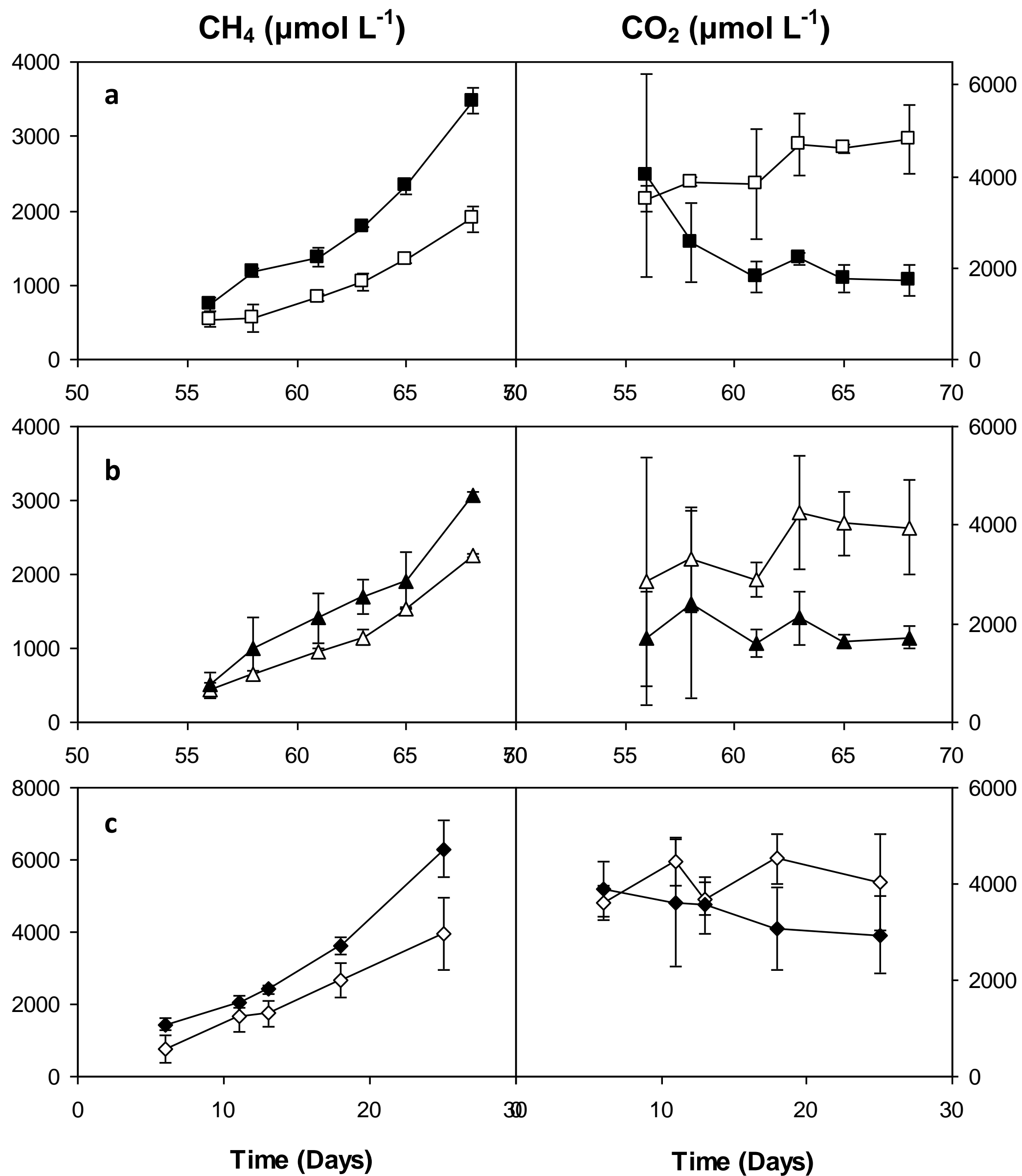

Figure 1 Methane and carbon dioxide production from SMFCs. Variation of methane and carbon dioxide concentrations (µmol L^{-1}) in pore water samples for MFC A (a) MFC B (b) and MFC C (c) with operation time. The values shown are averages from duplicate (MFC A and B) or triplicate experiments (MFC C). For each MFC constructed, three pore water samples were taken and the final concentrations were obtained by calculating the average. Error bars indicate standard deviation from duplicate (MFC A and B) or triplicate experiments (MFC C).

In order to elucidate the effect of SMFC on microbial processes, acetate concentration in pore water and stable carbon isotope ratios of methane ($\delta^{13}CH_4$) were determined for MFC-C. At day 6 of operation, pore water acetate concentrations were markedly lower in SMFCs than in open circuit controls suggesting that electrogenic bacteria were effectively degrading acetate to CO_2 and H^+ using the anode as terminal electron acceptor (Table S2). Reduced availability of acetate was corroborated by lower stable carbon isotope ratios of methane ($\delta^{13}CH_4$) in SMFCs (-57.7 ± 0.2 ‰ $\delta^{13}CH_4$ versus Vienna Pee Dee belemnite) compared to open circuit controls (-54.2 ± 1.2 ‰ $\delta^{13}CH_4$) (Fig. 2). The lower acetate availability in closed circuit SMFCs apparently induced a slight shift from acetoclastic to more hydrogenotrophic methanogenesis, the latter of which is known for strong discrimination against the heavy stable carbon isotope C-13 (Penning & Conrad, 2007) resulting in more negative $\delta^{13}CH_4$ values . The shift in the methane production pathway was reflected also in the composition of the archaeal community on anodes as analyzed by terminal restriction fragment length polymorphism (T-RFLP) and 16 rRNA gene cloning and sequencing (Fig. 3, Fig. S3 and Table S3). Acetoclastic *Methanosaeta* spp. (*in silico* terminal restriction fragment [T-RF] of 282-bp, Table S2) strongly decreased in relative abundance, whereas hydrogenotrophic Methanomicrobiales (*in silico* T-RF of 86-bp, Table S3) increased in anode samples from SMFCs compared with anodes from open circuit controls (Fig. 3 and Table 1). Principal component analysis (PCA) of T-RFLP data grouped anode samples from SMFCs and open circuit controls in separate clusters according to the abundances of the 86-bp and 282-bp T-RFs (Fig. S3), which was corroborated by 16S rRNA sequence analysis: SMFC anode samples had more clones affiliated to Methanomicrobiales and fewer affiliated to *Methanosaeta* spp. (Table S3).

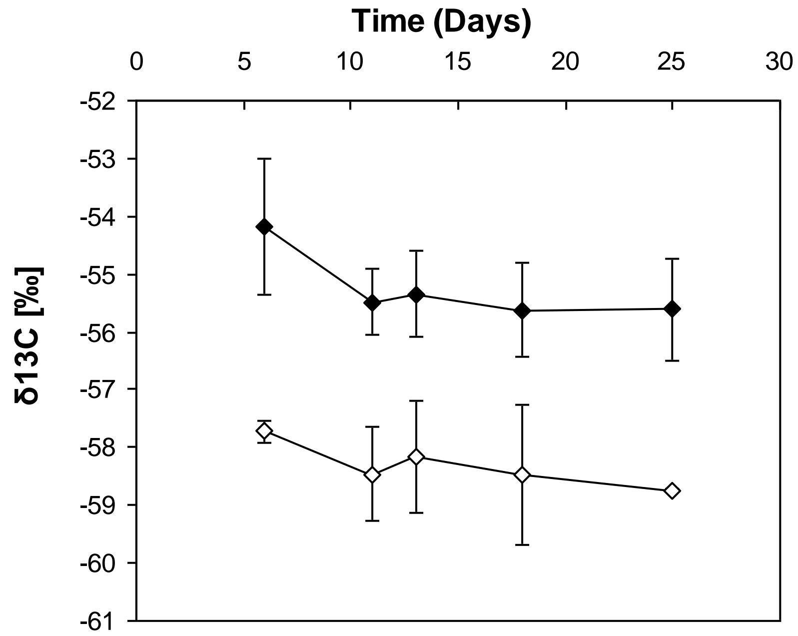

Figure 2. Stable carbon isotope ratios of methane ($\delta_{13}CH_4$).
Average of stable carbon isotope ratios of methane ($\delta_{13}CH_4$) for MFC C. Filled square: open circuit control; open triangle: sediment MFCs. The values shown are averages from triplicate experiments (MFC C). For each MFC constructed, three pore water samples were taken and the $\delta_{13}CH_4$ was obtained by calculating the average. Error bars indicate standard deviation from triplicate experiments (MFC C).

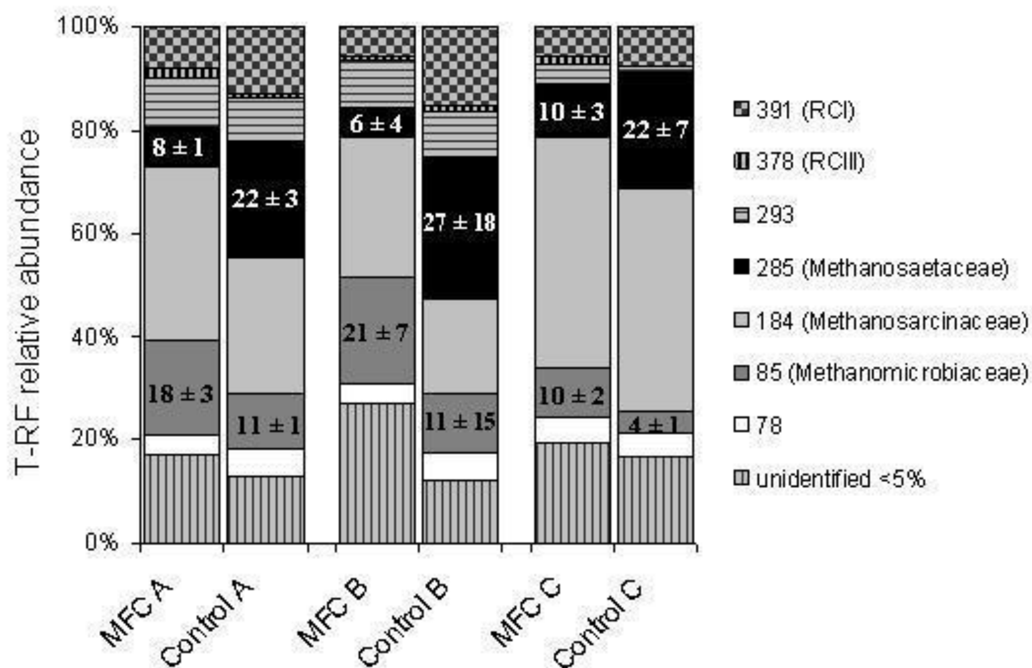

Figure 3 Archaeal community in rice field soil MFC assessed by T-RFLP. Average T-RF relative abundance of MFCs and controls are shown. Unidentified T-RFs with less than 5 % relative abundance were grouped. Error was calculated as the standard deviation between duplicate (MFC B) or triplicate (MFC C) experiments. For MFC A, the standard deviation was calculated for four anode samples, two samples per MFC, due to a higher anode surface.

In the present work we found that up to 1.2 µmol methane h^{-1} kg^{-1} soil were not formed in SMFCs with rice soil compared to open circuit controls. When we assume, that methane oxidation to CO_2 yields 8 electrons per mole, then each mole of methane not formed should equal a current of 263 nA kg^{-1} soil. The maximum current measured (4.85 nA kg^{-1} soil at 100 Ω), however, was ~100 fold lower than expected from the amount of methane that was not formed in closed circuit MFCs (Supplementary note S1). The lack in stoichiometry, i.e. current observed versus methane not formed, allows for two important conclusions: (1) the suppression of methane formation appears to be not simply based on competition for common electron donors and – most importantly – (2) MFCs have the potential to influence the electron flow in sediments to an extent that is significantly (here ~100 fold) beyond its capability to produce electrical current. The underlying mechanisms triggering suppression of methane production to such a large

extent are not understood yet. Since CO_2 production in closed circuit MFCs was higher than in controls (Fig. S2), it is unlikely that microbial activity was suppressed by MFCs. Thus, it appears that microorganisms capable of transferring electrons to the MFC anode had contributed to the increased CO_2 formation observed in closed circuit MFCs, most likely from acetate. Nevertheless, possible electron sinks other than the MFC anode must exist to explain the observed methane suppression but have not been identified yet. Sediment and soil bacteria have the capability to transfer electrons to a large number of soluble and insoluble electron acceptors including the anode of bioelectrical systems but the mechanisms involved in extracellular electron transfer are poorly understood (Rabaey*, et al.*, 2007). Electron transport over large spatial distances (>12 mm) has been detected recently in marine sediments (Nielsen*, et al.*, 2010) suggesting that electrical exchange exists between spatially separated biogeochemical processes. Similar mechanisms might be operative in our SMFC controlled rice field soil microcosms that could help explain the fate of electrons from organic matter oxidation not accounted by current stoichiometry. In our SMFC electrons might have been transferred via the anode to other electron acceptors present in rice field soil such as iron (III) or oxygen (present at the water-soil interface) thereby connecting two spatially separated zones of the sediment. These electrons would then not participate in current generation which might explain the lack of stoichiometry observed in our system.

Biogeochemical engineering approaches, such as controlling biogeochemical electron flow, might currently be a much more relevant application of sediment MFCs than the production of renewable energy. Our findings open the door for applications of microbial fuel cells to control biogeochemical processes that have a negative impact on climate (e.g., trace gas emissions) (Fig. 4). Other applications have not been explored yet but might be feasible as well, e.g., controlling biogenic sulphide or ammonia formation in production processes, large scale animal keeping, or acidification of mining lakes and waters.

6.5 References

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6.6 Supplementary data

Tables

Table S1 Electrochemical performance of sediment MFCs. Maximum and average current and power density values for the sediment MFCs are presented. Maximum values were obtained from the complete operation period; 75 days for MFC A and B, 35 days for MFC C. The average values were obtained from stable current production periods (23-70 days for MFC A and B and 17-35 days for MFC C).

	Maximum values		**Average values**	
	mA m^{-2}TAS	mW m^{-2}TAS	mA m^{-2}TAS	mW m^{-2}TAS
MFC A1	6.1	0.9	4.4	0.09
MFC A2	12.5	0.8	4.8	0.09
MFC B1	20.2	5.2	6.8	0.15
MFC B2	24.1	2.1	7.1	0.12
MFC C1	49.3	6.6	40.6	4.5
MFC C2	51.4	7.1	46.4	5.9
MFC C3	62.7	10.6	40.1	4.4

Table S2 Acetate and methane concentrations. Methane and acetate pore water concentration (μmol L^{-1}) for sediment MFC C, at day 6 of operation.

MFC	Acetate (μmol L^{-1})	CH_4 (μmol L^{-1})
MFC C1	ND	340
MFC C2	ND	1005
MFC C3	4.6	973
Control C1	14.7	1261
Control C2	10.6	1519
Control C3	4.6	1557

ND: Not detected, below the detection limit of 1 μmol L^{-1}.

Table S3. Archaeal community composition on anodes of Microbial fuel cells and open circuit controls. Archaea 16S rRNA clone libraries were constructed for anode of MFC and open circuit control. 16S rRNA sequence abundances were calculated from the number of clones obtained in Archaea clone libraries for anode samples from Control C (39 clone sequences) and MFC C (21 clone sequences). *In silico* T-RF were calculated for the restriction enzyme *Taq*I and T-RFs from the T-RFLP analysis were assigned to the different sequences found in the clone libraries. The clone sequence abundance was then compared with the T-RF abundance obtained from the T-RFLP analysis.

		Sequence abundance (%)		T-RF abundance (%)	
Taxonomic affiliation	*In silico* T-RF (bp)	Control	MFC	Control	MFC
Methanosarcina	184	51	48	43	44
Methanosaeta	282	33	14	22	10
Methanomicrobiales	84	5	19	4	10
Methanocella	391	3	5	7	6
RC-IV	390, 737, No cut	8	14	-	-

[a]Taxonomic affiliation was determined using RDP classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) and ARB parsimony tool.

Supplementary notes

Supplementary note S1 Efficiency calculation for MFC C. For the sediment MFC C, 93 µmol CH_4 L pore water^{-1} day^{-1} were not produced compared with the control (Table1). This corresponds to 1.2 µmol CH_4 Kg soil^{-1} h^{-1} considering the pore water content of rice field soil as 30 %. Methane oxidation to CO_2 yields 8 electrons per mole, then each mole of methane not formed should equal a current that can be calculated through the following equation:

$$I(A) = \frac{Q(C)}{t(s)} = \frac{molCH_4 8e^- F(Cmol^{-1})}{3600}$$

where I is the current in Amperes, Q the charge in coulombs, t the time in seconds and F the Faraday constant.

The methane losses observed equals a current of 263 nA per Kg of soil.

As the maximum current measured was 4.85 nA per Kg of soil, a maximum of 1.8 % of the theoretical current is obtained.

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Chapter 7

General Discussion and Perspectives

In this work, diversity and function of microorganisms on anodes of SMFC fueled by rice root exudates were studied. This type of SMFCs was developed in 2008 however, up to date, scarce data on the anode microbiology is available. This work focused on the study of the factors determining the anode bacterial communities and the identification of potential anode reducers (**chapters 3 and 4**), the identification of bacteria degrading rice root exudates on anodes and possible carbon flow (**chapter 5**) and the effects of SMFCs on methane emission from rice paddy soils (**chapter 6**).

7.1 Microbial diversity on anodes from SMFCs fueled by rice root exudates

Factors influencing microbial communities on anodes

Rhizosphere bacterial community compositions are likely to be determined by many different selection factors that influence the growth and size of different bacterial populations. This includes the composition and quantity of root exudates and other carbon substrates provided by rhizodeposition (Hartmann*, et al.*, 2009). In planted sediment microbial fuel cells (SMFC) an additional selection factor emerges: the anode as alternative electron acceptor. In **chapters 3 and 4** we showed that the support used for plant growth is an important factor determining bacterial and archaeal community compositions on anodes. Bacterial and archaeal community compositions on anodes of planted sediment microbial fuel cells (SMFC) differed when analyzed with terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel

electrophoresis (DGGE). Cluster analysis showed that samples clustered mainly according to the support used (vermiculite, potting soil and rice field soil). An explanation for this could be the different indigenous microbial populations existing in the different support types. It has been reported that soil characteristics such as pH, O_2 concentration, physico-chemical characteristics (Cavigelli*, et al.*, 1995, Gelsomino*, et al.*, 1999, Carelli*, et al.*, 2000, Liesack*, et al.*, 2000, Ludemann*, et al.*, 2000, Noll*, et al.*, 2005), carbon availability and mineral content (for example N and Fe) (Hu*, et al.*, 1999, Degens*, et al.*, 2000, Rui*, et al.*, 2009) are factors which affect the microbial community composition in soils. The influence of soil type (river sediment *vs.* rice field soil) on the archaeal community composition on rice rhizosphere has been reported previously (Conrad*, et al.*, 2008). When rice plants grew on rice field soil, the methanogenic community was dominated by Rice Cluster 1 (RC-1) while when grown on river bank soil, roots were colonized by Methanomicrobiales. This affected methane emission which was higher when RC-1 colonized the roots which demonstrated that the type of soil has a potentially important impact on the methane cycle. In SMFCs different anode bacterial communities were detected when marine or freshwater sediments were used (Holmes*, et al.*, 2004). The composition of Bacteria and Archaea inhabiting the support which is used for the construction of the planted SMFCs is important and will determine the microbial community that will develop on the anode. In two chamber MFCs the inoculum affects the internal resistance of the system (Ieropoulos*, et al.*, 2010) as well as the power output and the biofilm adhesion (Jiang*, et al.*, 2010). However, the effect on the bacterial community composition has not been thoroughly investigated. When comparing the anode microbial communities reported by different research groups different bacterial community compositions were detected when different inocula were used (Table 2 in section 1.1.4.1). However, different MFC configurations, anode materials and operation conditions were used which are also likely to affect the anode microbial community compositions.

In SMFCs fueled by rice root exudates, the natural support to be used for real application purposes is rice field soil. The rice field soil bacterial community adapted to environmental conditions of rice fields might favor SMFC current production by decreasing lag phases and by producing stable current outputs for long periods. It has been shown that in rice rhizosphere, the microbial community at two different time

points (45 and 90 days) changed probably due to changes in the root exudation pattern (Lu*, et al.*, 2006). An anode biofilm which quickly adapts to new environmental conditions might improve the efficiency of the system. In our work (**chapters 3 and 4**), higher current was observed when potting soil was used as support for the plant, however, also when comparing unplanted controls which could be due to a higher organic matter content of potting soil (20%) compared to rice field soil (1.5%). The increase in current density by the addition of a rice plant was in the same order (2-4 times higher in planted SMFCs compared to unplanted controls). Stable current production in the SMFCs with rice field soil were observed after 25 days of operation (**chapter 5**) while in the SMFCs with potting soil stable current outputs where only attained after approximately 50 days of operation (**chapter 2**). The configuration and operation condition of the SMFCs slightly differed which might have affected the current production. Comparison of anode microbial community compositions of SMFCs with equal configurations and operation conditions, using different soils as support for the plant would allow getting more insight into the effect of soil type on current production. Moreover, the study of the anode bacterial community compositions at different time points would enable to determine changes in the anode reducing bacteria with time and the influence on current production. Planted SMFC could also be applied to harvest energy from wetlands; microbial community compositions on anodes of wetland SMFC fueled by root exudate would allow to get further insight into diversity and function of anode biofilms.

In **chapters 2 and 3** we also studied the effect of the plant on the anode microbial community composition. The influence of the plant was less apparent (however detectable) than the influence of the support used as discussed in **chapter 3**. Similar results were obtained by Marschner (2001) where the bacterial community of rhizosphere was influenced more by the soil type than the plant species. Interestingly, the influence of the plant on the microbial community was higher when supports with low carbon content like vermiculite were used and lower in potting soil where the carbon content was the highest (20%). The study of the influence of different rice plant varieties and root exudate compositions on the anode bacterial community when rice soil is used would contribute to further understand the relation between the anode microbial

community and the plant. This might be important for future application of this technology in real rice fields.

Diversity of potential anode reducing bacteria

In **chapters 4 and 5**, we showed that the support type selected the potential anode reducing bacteria responsible for current production. The type of anode reducing bacteria enriched on the anode might influence the efficiency of the planted SMFCs. The two soils used as support selected for two main different groups of anode reducing bacteria, *Desulfobulbus* related bacteria with potting soil and *Geobacter* spp. with rice field soil. As discussed in **chapter 3**, this is probably translated into different current producing mechanisms: *Desulfobulbus* spp. might transfer electrons involving sulfur compounds or volatile organic acids other than acetate and *Geobacter* spp. probably through the oxidation of acetate. It has been reported that current generation by bacteria with an oxidative metabolism is more effective in current production than by bacteria with a fermentative metabolism where only one third of the electrons are available for current production (Rabaey & Verstraete, 2005). Moreover, it has been reported that *Geobacter sulfurreducens* was able to produce electrical power at levels that are comparable to those observed in mixed culture microbial fuel cells (Nevin*, et al.*, 2008). Would then biofilms with *Geobacter sulfurreducens* produce more efficient systems? What is the bacterial composition of the most efficient biofilm? In our work, higher currents were obtained with potting soil (44mA m^{-2} TAS *vs.* 8 mA m^{-2} for potting soil and rice soil respectively) which would indicate that anode biofilms with *Geobacter* spp. did not produce higher currents. However, the higher current production with potting soil might have been due to higher organic matter content (20% vs. 1.5% for potting soil and rice soil respectively) or due differences in the configuration and operations. The indigenous microbial composition present in the support to be used will determine the main current producing bacteria of the system, the current producing mechanism and probably the fuel cell efficiency.

In the rice soil SMFCs studied several unknown potential anode reducing bacteria were abundant on anodes such as *Anaeromyxobacter* related spp. and unclassified δ-Proteobacteria within δ-Proteobacteria and Anaerolineae within Chloroflexi. In two-

chamber MFCs and single-chamber MFCs, several potential anode reducing bacteria have been found (Table 2 in chapter 1). However, isolation strategies and the operation of pure culture MFCs are needed to determine the ability of these bacteria to transfer electron to an anode and the mechanisms involved. These pure culture studies would help in relating bacterial identities with function in other MFCs. *Anaeromyxobacter*, unc. δ-Proteobacteria and Anaerolineae could also be involved in degradation of root exudates and would then play a relevant role in the anode biofilm by providing the electron donors for anode reducing bacteria (Discussed in **chapter 6**). Furthermore, a recent study (Strycharz et al, 2010) shows that *Anaeromyxobacter dehalogenans* can use the cathode as electron donor for reductive dechlorination of 2-chlorophenol indicating the ability of *A. dehalogenans* to interact with electrodes.

In **chapter 4**, we detected distinct clusters within the genera *Geobacter* and *Anaeromyxobacter* that were stimulated by root exudates. The diversity within the genus *Geobacter* in MFCs and the factors determining the selection of certain species has not been addressed. Determining the factors affecting competition within *Geobacter* spp. might contribute to select for specific high current producing species. Several potential anode reducing populations were detected on anodes of planted SMFCs. Whether these bacteria are competing for a common electron donor or if they are positively interacting to improve current production, remains unanswered.

7.2 Model for carbon flow on anodes

One of the major tasks of microbial ecology is linking microbial diversity with function of ecosystems (Dumont & Murrell, 2005). By using RNA-stable isotope probing (RNA-SIP) we contributed in understanding the microbial processes occurring on anodes of rice planted SMFC (**chapter 5**). The microenvironment surrounding the anode of planted SMFCs is of higher complexity than in “normal” SMFC anodes due to the diversity of organic compounds available from root exudation. Root exudates are composed of a diverse set of organic compounds; carbohydrates, aminoacids, amides, aliphatic acids, aromatic acids, fatty acids, sterols, enzymes, hormones, vitamins, and others (Grayston, *et al.*, 1997). In **chapter 5** we showed that even though the release of rice root exudates into the anode surrounding increased current production, the main current producers

were not actively involved in degrading photosynthetically fixed carbon as δ-Proteobacteria did not become labeled. However, we cannot dismiss the possibility that some of the bacteria actively degrading root exudates might be able to use the anode as final electron acceptor. We suggest four possible processes which could be occurring in parallel on the anode (Fig. 1):

A) Current production from acetate. Acetate is a key intermediate in the degradation of organic matter in sediments. We found the following evidences for this hypothesis: a) *Geobacter* spp. were the most active potential anode reducing bacteria found on the anode by cloning/sequencing of 16S rRNA (**chapters 4 and 5**), b) *Geobacter sulfurreducens* and *Geobacter metallireducens* are able to produce current from acetate in pure culture (Bond*, et al.*, 2002, Bond & Lovley, 2003), c) Acetate concentration in planted SMFCs were 10 fold less than in open circuit controls (**chapter 5**) and d) Methanosaetaceae, acetotrophic methanogens, were drastically reduced on anodes of planted SMFC compared with open circuit controls (**chapters 3, 4 and 6**).

B) Root exudate degradation without current production. Root exudates are released by the plant into the soil and are actively degraded by rhizospheric bacteria. This process might also occur on anodes of planted SMFCs as suggested by the following result: several bacteria were found labeled both in the open circuit control, where no current is produced, as well as in planted SMFCs (e.g. *Dechloromonas* spp., Anaerolineae (114-bp T-RF), unclassified Kineosporiaceae (136-bp T-RF)) (**chapter 5**).

C) Direct current production from root exudates. Current was enhanced by root exudates however; can they be used directly for current production? We found evidence that some bacteria might be coupling root exudate degradation with current production: a) Unclassified *Geobacter* (cluster 1) and *Anaeromyxobacter* spp. (cluster 4B) were stimulated by rice root exudates (**chapter 4**), b) Unclassified δ-Proteobacteria (cluster 5) were only detected in the planted SMFC but not in unplanted and open circuit controls (**chapter 4**), c) Some labeled bacteria were only detected in the planted SMFCs and not in the open circuit control (e.g. T-RFs of 510-bp and 214-bp) (**chapter 5**), d) some labeled bacteria have been found previously on anodes from MFCs (e.g. *Acidovorax* spp. (Phung*, et al.*, 2004, Kim*, et al.*, 2006, Borole*, et al.*, 2009, Lefebvre*, et al.*, 2010) and

e) some labeled bacteria were more abundant in the planted SMFCs than in open circuit controls (e.g. Oxalobacteraceae) (**chapter 5**).

D and E) Current production from soil organic matter or acetate derived from soil organic matter degradation. Current is produced also in unplanted SMFCs probably by the degradation of soil organic matter into acetate. Is soil organic matter also used as fuel when root exudates are produced? It is likely that degradation of soil organic matter occurs on the anode as *Clostridium* spp., associated with degradation of polymers in rice field soil (Liesack*, et al.*, 2000), were still active (**chapters 4 and 5**). Acetate produced from the degradation of soil organic is likely to be used by anode reducing bacteria. Whether soil organic matter is used directly for current production could not be determined.

The microbial processes on anodes that finally result in current production seem complex and interaction of several bacteria populations is necessary.

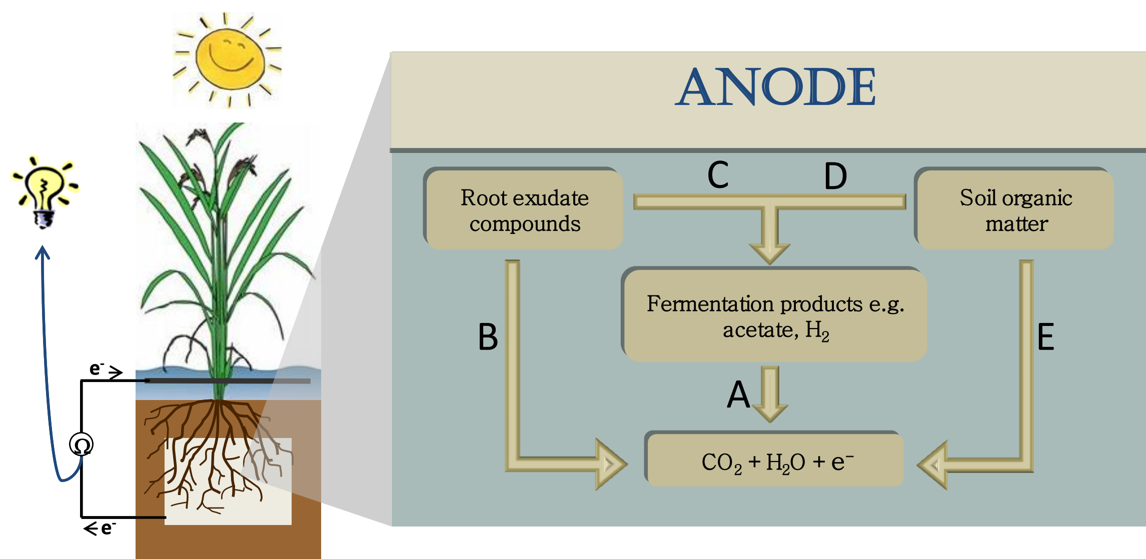

Figure 1 Scheme depicting the possible fuels for current production in planted SMFCs. Methanogenesis is not included in the scheme. Organic matter (root exudates (C) and soil organic matter (D)) is degraded and finally converted to acetate (or other fermentation products). Anode reducing bacteria might use acetate (A), root exudates (B), or other organic compounds present in soil organic matter for current production (E).

7.3 SMFCs as methane emission mitigation strategy

Rice paddies are a major source of the greenhouse gas methane contributing up to 15% to the global atmospheric emission budget (Intergovernmental Panel on Climate Change-IPCC, 2007). The search for methane emission mitigation strategies is highly relevant as the methane budget will even increase in future in correlation with the food demands of the growing human world population (IPCC, 2007). In **chapters 3 and 4**, we reported changes in the archaeal community compositions on anodes with a decrease of the acetotrophic methanogen *Methanosaeta*. In **chapter 6**, we were able to show for the first time that anodes introduced into rice paddy soil (Fig. 2) were able to reduce methane emission by almost 50%. The mechanism undergoing this process is not clear and the competition for acetate alone could not explain the decreases in methane emission observed.

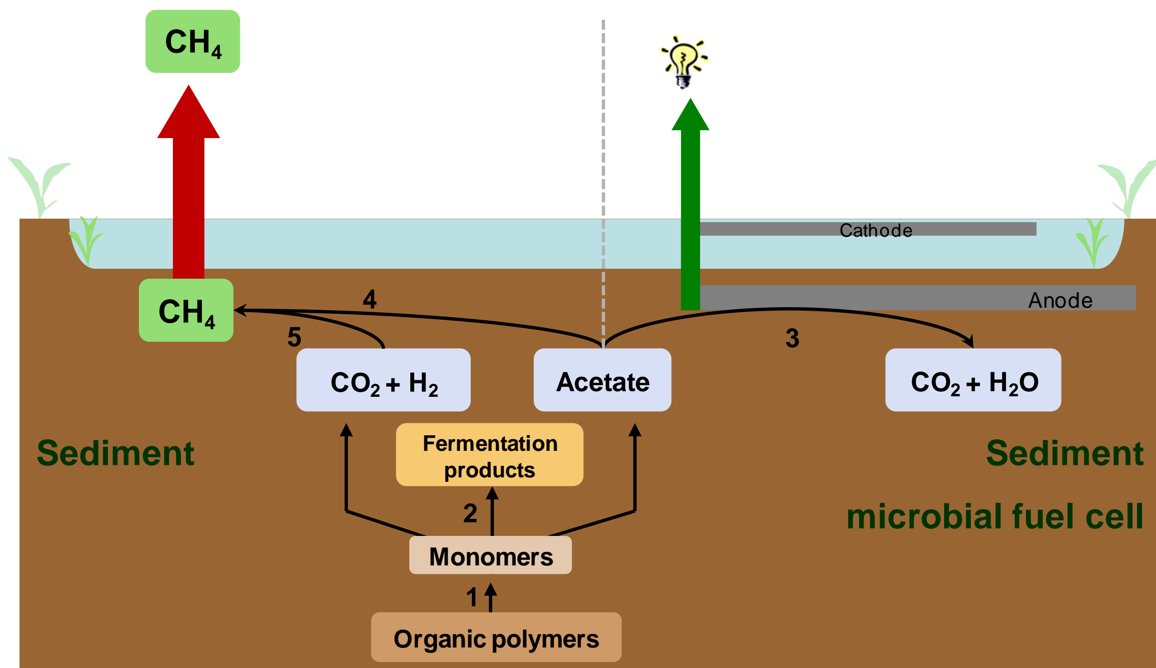

Figure 2 Taming Methane emissions using sediment microbial fuel cells. Schematic figure showing differences in the degradation of organic matter and consequently in the amount of methane emission between a sediment and a sediment microbial fuel cell (SMFC). Common reactions for both sediments and SMFCs: 1. Depolymerization 2. Primary fermentation 5. Hydrogenotrophic methanogenesis. In the SMFCs, acetate will be respired using the anode as final electrode acceptor (3) while in a sediment the acetate would be used by acetotrophic methanogenesis and converted into methane (4). Acetotrophic methanogenesis probably also occur in SMFCs but is less important than in a sediment.

Electron transport over large spatial distances (>12 mm) has been detected in marine sediments (Nielsen*, et al.*, 2010) suggesting that electrical exchange exists between spatially separated biogeochemical processes. We suggest that the anode could be connecting spatially separated zones and electrons transferred to the anode by anode reducing bacteria in one zone might migrate through the anode to a second zone participating in for example reduction of iron (III) or oxygen (Fig. 3).

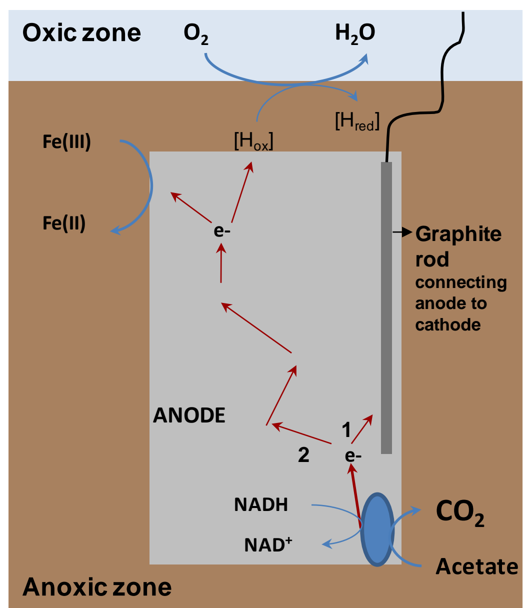

Figure 3 Scheme depicting the migration of electrons thorough the anode. Anode reducing bacteria oxidize acetate and transfer electrons to the anode. The electrons could then generate a current (1) by entering the circuit via the graphite rod connecting to the anode with the cathode or 2) migrate through the anode to a different zone of the sediment participating finally in a different electrochemical process like for example reduction of oxygen or iron. [H] refers to electron shuttle molecule.

This would not only explain the lack of stoichiometry found (**chapter 6**) but also the presence of anode reducing bacteria like *Geobacter* in open circuit controls (**chapters 4 and 5**). The microbial community detected on anodes of open circuit controls differed from the bulk soil microbial community. This could be due to 1) the preference of some bacteria to use the anode as support for growth, 2) the use of the anode as electron acceptor without current production and 3) higher influence of rice root exudates on the anode compared to the bulk soil (higher root:anode relation compared with root:bulk

soil relation) changing the microbial community composition. Further experiments would be needed to fully unravel the processes occurring on anodes of open circuit controls.

Methane can be transported to the atmosphere by several mechanisms, for example via diffusion through the soil and water; however, the main fraction of methane is transported through the root aerenchyma system of the plant (Dubey, 1995). In wetland soils, plant-derived organic substances serve as an important carbon source for CH_4 production and emission (Dannenberg & Conrad, 1999, Kimura*, et al.*, 2004). The addition of a rice plant into the system would increase the available electron donors (for example acetate) for methanogenesis and electrogenesis. This could affect the efficiency of the mitigation effect of a SMFC anode on methane production from acetate. Moreover, Kaku *et al.* (2008)did not succeed in reducing methane emission on a real rice field soil with sediment microbial cells. Therefore methane emission mitigation in rice planted SMFCs should be studied in order to confirm the importance of this mitigation strategy.

7.4 Perspectives

Our study on microbial diversity and function on anodes of rice planted SMFCs is a first step in understanding the anode biofilm and current production in these systems. We were able to determine that the support used for the plant is an important factor for determining the bacteria that will develop on the anode. However, the importance of the inoculum should be investigated more rigorously, both in SMFCs as well as in other MFC setups, comparing anode biofilms developed under the same conditions. The effect of the bacterial community composition of the anode biofilm in current production should also be further investigated in order to determine whether the presence of certain species might enhance current production. Another interesting point which still needs to be unraveled is the interaction (or competition) between the different bacteria found on anodes. Moreover, the competition of different anode reducing species might help to understand more about the current generation mechanisms in MFCs. The study of the labeled Archaea on anodes would also contribute to further understand the role of Archaea on the anode. Regarding methane emission, as mentioned above, it would be of great importance to demonstrate that SMFC anodes also decrease methane emissions in planted SMFCs.

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Chapter 8

General Conclusions

In the present PhD thesis a novel ecosystem was studied namely the anode of rice soil SMFCs fueled by rice root exudates. The techniques used, T-RFLP, cloning/sequencing of 16S rRNA, Stable Isotope Probing and 454-pyrosequencing allowed for the study of microbial diversity and function of anode bacteria. We identified the main factors affecting the anode biofilm composition, the potential anode reducing bacteria as well as the anode bacteria actively using photosynthetically fixed carbon. Soil type was the main factor determining the anode reducing community compositions and the relevant current producing bacteria. *Geobacter* spp. and *Desulfobulbus* related spp. were the main current producing bacteria in rice soil and potting soil planted SMFCs, respectively. Novel potential anode reducing bacteria were detected on anodes: deltaproteobacterial *Anaeromyxobacter* related spp., Anaerolineae and unclassified δ-proteobacteria. Moreover, within the genus *Geobacter*, different clusters were stimulated in planted and unplanted SMFCs which suggest that competition between different species exist. In planted SMFCs, current is probably obtained by the interaction of a highly diverse microbial community where degradation of organic matter as well as oxidation of acetate by anode reducing bacteria are important processes. Finally, we proposed a novel application of SMFCs: methane emission mitigation from rice field soils. Up to 50% methane emission decrease was observed in SMFCs with rice field soils. Our data might contribute with the knowledge of the diversity and function of anode reducing bacteria and how SMFCs affect methane emission from rice field soils.

Appendices

Photos of setups

Figure 1 Example of an anode made by carbon felt, joined to the cupper cable by a graphite rod.

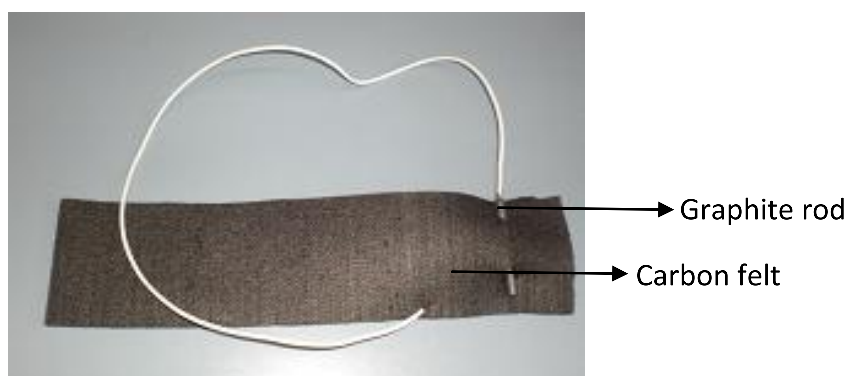


Figure 2 Sediment microbial fuel cell used for studying the methane emission reduction. Anode is embedded in the rice soil and cathode in the overlying water. Oxygen is sparged into the overlying water.

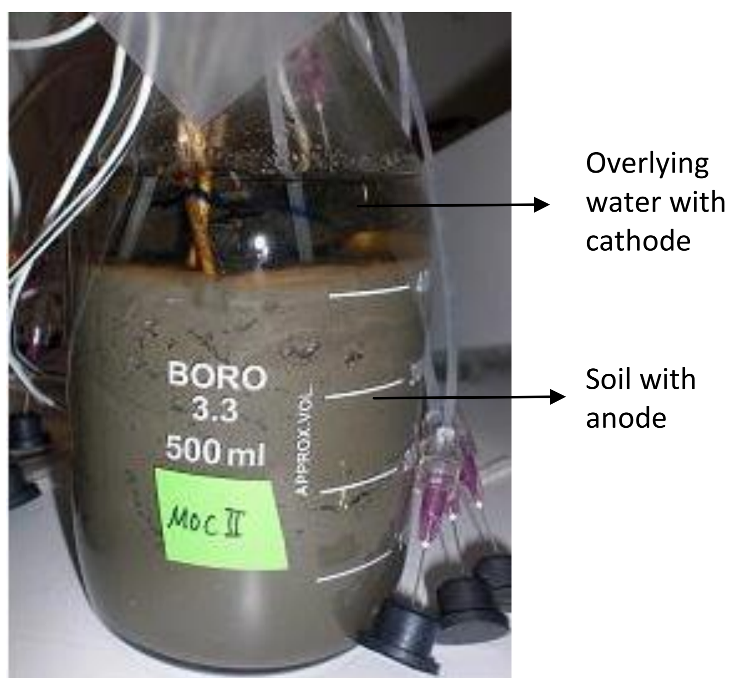
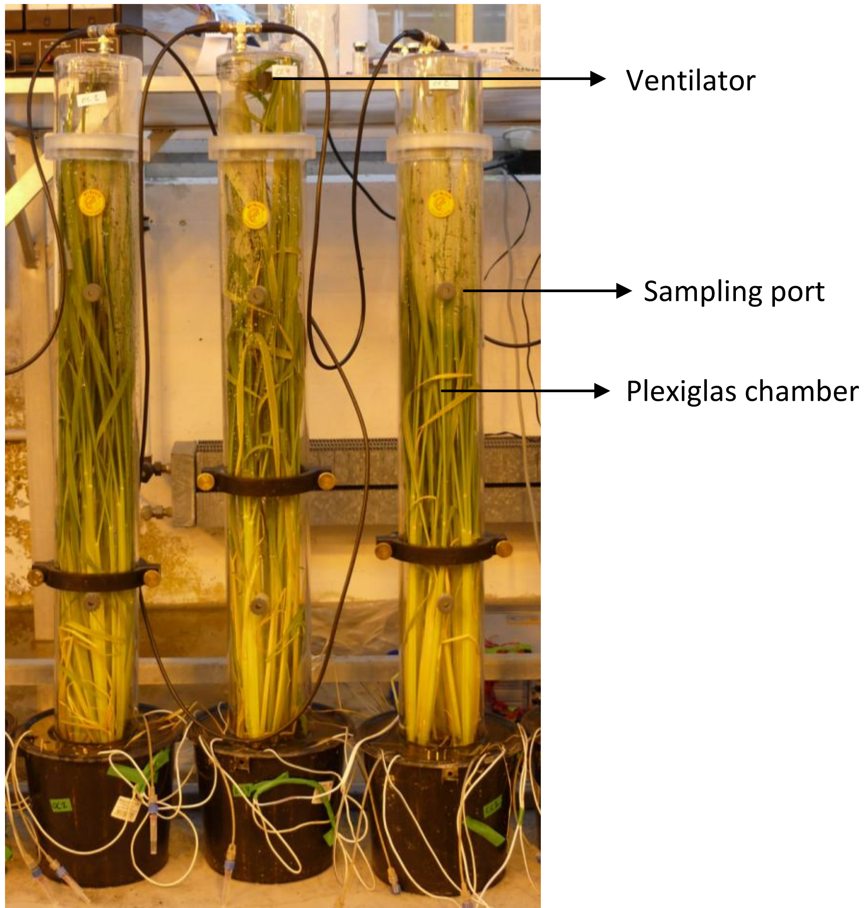


Figure 3 Planted sediment microbial fuel cells. A) Planted sediment microbial fuel cells in the greenhouse. Datalogger records potential from the different setups. b) Connection of anode and cathode through a resistance protected from humidity.

Figure 4 Harvesting of anodes and cathodes a) sediment microbial fuel cell after removal from plastic container showing cathode, roots and soil. b) Position of the anode in the sediment microbial fuel cell c) removal of anode from sediment microbial fuel cell

Figure 5 Stable isotope labeling with ^{13}C-CO2. Plants were covered with a Plexiglas chamber with two ports for taking samples and one ventilator.



List of abbreviations

16S-rRNA	small subunit of the ribosomal RNA
6-FAM	6-Carboxyfluoresceine
bp	base pairs
BSA	Bovine serum albumin
CC	Closed circuit
DGGE	Denaturing Gradient Gel Electrophoresis
EDTA	Ethylenediaminetetraacetic acid
FAM	Carboxyfluorescein
FID	Flame ionization detector
GA	Geometric area
GC	Gas chromatography
HPLC	High performance liquid chromatography
IRMS	Isotope ratio mass spectrometer
MFC	Microbial fuel cell
NA	Nucleic acids
NP	Unplanted
OC	Open circuit
PCA	Principal component analysis
ppm	parts per million
SMFC	Sediment microbial fuel cell
TAS	Total anode surface
TNS	Tris, HCl, SDS buffer
TRF	Terminal restriction fragment
TRFLP	Terminal restriction fragment length polymorphism
δ13C	Stable carbon isotope ratio relative to the international standard

Curriculum Vitae

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Education

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Publications in peer reviewed journals

De Schamphelaire L, Cabezas A, Marzorati M, Friedrich MW, Boon N, Verstraete W (2010) Microbial community analysis of anodes from sediment microbial fuel cells powered by rhizodeposits of living rice plants. Appl Environ Microbiol 76: 2002-2008.

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Conference presentations

VAAM meeting (2009) Breidenbach B, Cabezas A, Friedrich MW. Effect of sediment microbial fuel cells on methane emission from rice paddies

Workshop on electrochemically active biofilms (2008) Cabezas A, de Schamphelaire L, Boon B, Verstraete W, Friedrich MW. Microbial diversity in Sediment Microbial Fuel Cells fueled by rice root exudation

107th American Society for Microbiology meeting (2007) Draper P, Cabezas A, Crolla I, Etchebehere C. Evaluation of different strategies to isolate denitrfiers from an industrial wastewater treatment reactor

VAAM meeting (2007) Jetten M, Harhangi H, Kartal B, Cabezas A, van de Vossenberg J, Op den Camp H, Strous M. Metabolic versatility of anammox bacteria

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VIII Latin American Workshop and Symposium on Anaerobic Digestion (2005) Draper P, Cabezas A, Etchebehere C. A change in the microbial population causes ammonification in denitrifying reactors

VIII Latin American Workshop and symposium on Anaerobic digestion (2005) Benítez A, Ferrari A, Gutierrez S, Canetti R, Cabezas A, Travers D, Menes J, Etchebehere C. Sequencing Batch Reactors as a post treatment on anaerobically treated dairy effluent

VIII Latin American Workshop and symposium on Anaerobic digestion (2005) Cabezas A, Draper P, Muxí L, Etchebehere C. Post-treatment of a Slaughterhouse wastewater - stability of the microbial community of a sequencing batch reactor operated under oxygen limited conditions

10th World Congress on Anaerobic Digestion (2004) Cabezas A, Etchebehere C, Muxí L. Quantification of autotrophic nitrifying bacteria in wastewater treatment systems

Ninth International Symposium on Microbial Ecology (2001) Etchebehere C, Cabezas A, Errazquin MI, Muxí L. Characterization of denitrifying strains isolated from anoxic reactors by ARDRA profiles

Ninth International Symposium on Microbial Ecology (2001) Etchebehere C, Cabezas A, Errazquin MI, Muxí L. Evaluation of the methanogenic and denitrifying consortium in an anaerobic treatment lagoon

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Pledge

I certify that the present thesis entitled:

"Diversity and Function of the Microbial Community on Anodes of Sediment Microbial Fuel Cells fueled by Root Exudates"

Was carried out without any unlawful means; no literature resources, reagents and technical devices were used other than those stated. This work has never been submitted before in this or similar format to any other university and has not been used before any examination.

Marburg, October 2010

Angela Cabezas da Rosa